Investigating the Mechanism of Action of Colostrinin™ on Cells in Culture

Thesis

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Investigating the mechanism of action of Colostrinin™ on cells in culture

A thesis submitted for the degree of Doctor of Philosophy

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Abbreviations

4HNE:	4-hydroxynonenol
ABDS:	Antibody diluting solution
Ach:	Acetylcholine
AD:	Alzheimer's disease
AICD:	APP intracellular domain
ALS:	Amyotrophic Lateral Sclerosis
ApoE:	Apolipoprotein E
APP:	Amyloid precursor protein
Aβ:	Beta-amyloid
BME:	Basal Medium Eagle
Cdk5:	Cyclin dependent kinase 5
c-JNK:	c-Jun-terminal kinase
CLN:	Colostrinin™
CNPase:	2, 3-Cyclic nucleotide 3-phosphodiesterase
CNS:	Central Nervous System
CSF:	Cerebrospinal fluid
CVNP:	Colostral-Val nonapeptide
DCF:	Dichlorofluorescein
DCFH-DA:	2'7' Dichlorofluorescein diacetate
***DIV*:**	Day *in vitro*
DMEM:	Dulbecco's Modified Eagle's Medium
E18:	Embryonic day 18
EBSS:	Earl's Balanced Salt Solution
EM:	Electron microscopy
FACS:	Fluorescence Activated Cell Sorting
FAD:	Familial Alzheimer's disease
FCS:	Fetal calf serum
GABA:	Gamma Aminobutyric Acid
γ IFN:	Gamma interferon
GFAP:	Glial fibrillary acidic protein
GSK3β:	Glycogen synthase kinase 3 beta
H_2O_2:	Hydrogen peroxide
HRT:	Hormone replacement therapy
HS:	Horse Serum
LDL:	Low Density Lipoprotein
LPS:	Lipopolysaccharide
LTD:	Long Term Depression
LTP:	Long Term Potentiation
MAP-2:	Microtubule associated protein 2
MCI:	Mild cognitive impairment
MMSE:	Mini Mental State Evaluation
MS:	Multiple sclerosis

MTS:	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium
n/mAChR:	Nicotinic/muscarinic Acetycholine receptor
NFT:	Neurofibrillary tangles
NO:	Nitric Oxide
NTR:	Neurotrophin receptor
P4:	Postnatal day 4
PBS:	Phosphate buffered saline
PD:	Parkinson's disease
PDGF:	Platelet derived growth factor
PDNF:	Platelet derived neurotrophic factor
PI3K:	Phosphoinositide 3 kinase
PKA/C:	Protein kinase A/C
PNA:	Peanut agglutinin
PS:	Presenilin
ROS:	Reactive oxygen species
RT:	Room temperature
SOD:	Superoxide dismutase
TNFα:	Tumour necrosis factor alpha
TUNEL:	Terminal deoxynucleotidyl Transferase (TdT) mediated dUTP nick end labelling

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Abstract

The aim of this Ph.D. project was to investigate the effects of Colostrinin (CLN) on cells in culture, and its ability to prevent or alleviate cytotoxicity induced by reactive oxygen species (ROS) and beta-amyloid (Aβ). Two cell culture systems were used to analyse the effects of CLN; rat primary hippocampal cultures and the B50 rat neuronal cell line.

Bovine CLN was found to have no adverse effects on cell morphology or survival and to have a small, non-significant effect, on both menadione-induced, oxidative stress-mediated toxicity and $A\beta_{1-42}$-induced toxicity of neurons in primary hippocampal cultures. This protective effect was found to potentially be related to the antioxidant effects of CLN. CLN was demonstrated to prevent a menadione-induced increase in ROS in the B50 cell line. Furthermore CLN was able to reverse a significant $A\beta_{1-42}$-mediated increase in the protein levels of the antioxidant enzyme Cu/Zu superoxide dismutase (SOD) in primary hippocampal cultures, although CLN alone caused an increase in SOD1 protein in the B50 cell line.

Bovine CLN was shown, in both B50 cells and primary hippocampal cells, not to significantly decrease the protein levels of cyclin dependent kinase 5 (Cdk5) which has previously been demonstrated to be involved in the mechanism of tumour necrosis factor (TNF) α-mediated protection against Aβ-induced toxicity in primary hippocampal cultures. Furthermore CLN did not consistently alter the expression of activated caspase 3 in either B50 or primary hippocampal cells.

This study adds to the knowledge and understanding of the mechanism of action of CLN.

Chapter 1

Introduction

In this introduction I will briefly summarise the cellular features exhibited in Alzheimer's disease (AD) as well as some of the other main neurodegenerative diseases in order to illustrate some of the similarities and differences in the pathology and causative agents of these diseases. I will discuss the physiological processes of oxidative stress and apoptosis with a focus on how these are altered in pathology, most specifically in AD. Finally, I will review the literature on Colostrinin (CLN), focusing on its structure, its effects on the immune system and oxidative stress and evidence of its potential therapeutic effects in AD patients.

There are a number of neurodegenerative diseases of the central nervous system (CNS) including AD, Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and Huntington's disease. These diseases have some common factors as they all involve cell loss within the CNS and protein aggregation in some form. Oxidative stress is also a common feature of these diseases and will be discussed in detail towards the end of this introduction. However there are also many differences in the proteins involved, cell types affected and underlying causes of the pathology of these different diseases. These differences lead to a spectrum of diseases which have similarities but may vary in age of onset and the rate of disease progression as well as the specific symptoms which may include cognitive impairment, or effects on motor control and movement.

1.1. Alzheimer's disease

As detailed in the previous section a variety of neurodegenerative diseases exist. There are also several different types of dementia which manifest in various ways, including PD, Dementia with Lewy Bodies, AD, Creutzfeldt-Jakob disease, frontal lobe dementia, vascular dementia and alcohol-related dementia. For example Creutzfeldt-Jakob disease is characterised not only by rapidly progressive dementia but also commonly cerebellar or visual disturbance (Knight and Will 2004) and vascular dementia leads to deficits in executive function, however, the main decline seen in AD patients is in memory loss (Kalaria 2002). These diseases have different causes and may affect different types of cognitive functioning dependent on which areas of the brain are affected. Of these AD is the most common cause of dementia in the elderly, affecting around 18 million people worldwide (Farfara *et al.* 2008). At present there is no cure for AD and the only treatment is of the symptoms resulting from loss of transmitters, mainly acetylcholine (Palmer 2002).

According to the US Fisher Centre for Alzheimer's Research Foundation, about 10 percent of people aged 65 and over, are affected by this disease and the prevalence doubles roughly every 10 years after age 65 (US Fisher Centre for Alzheimer's Research Foundation). Half of the population aged above 85 years may have AD or exhibit symptoms to varying degrees of progression or severity. Cognitive deficits arising from AD increase as the disease progresses and although AD does not in itself result in death, the decline in cognitive function and ability to look after oneself leads to malnutrition and general ill health with eventual death. Death typically occurs within 10 years of the diagnosis of the disease in the late onset form of AD which manifests after the age of 65.

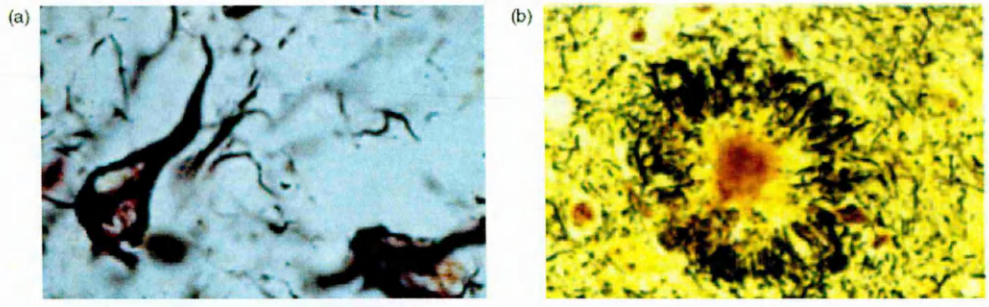
The first descriptions of AD are reported to have come initially around 100 years ago from Dr. Alois Alzheimer who had followed the disease progression of a

lady referred to as Auguste D (Alzheimer *et al.* 1995). He made detailed drawings of his discoveries showing the now well- known pathological hallmarks of the disease. Despite Alzheimer being credited with discovery of the disease, pathology had been mentioned by others prior to this and since then these observations have been seen and confirmed in numerous cases of dementia.

Clinically AD manifests itself almost entirely as a cognitive disease with little in the way of physical disability. The cognitive decline begins as minor memory problems that may only be obvious to the patient themselves and progresses to full dementia. Various aspects of this dementia have been studied because an increase in the understanding of the exact causes of the deficit will allow more efficient treatment regimens and aid diagnosis. From one of these studies, using tests to specifically test structural and semantic knowledge of the same objects, it has been found that deficits in semantic and structural knowledge may not be connected (Hajilou and Done 2007). Furthermore aesthetic preferences have been shown to remain relatively intact despite the cognitive decline as evidenced by the fact that AD patients have shown the same stability in their art preference as normal controls (Halpern *et al.* 2008).

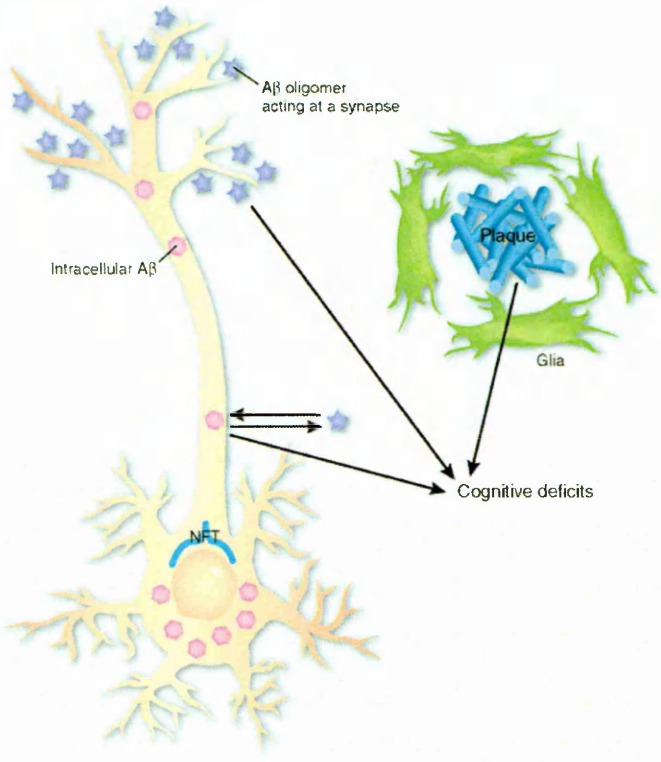
1.1.1. Pathology

Pathologically, AD is characterised by the presence, usually observed at post-mortem, of extracellular senile plaques consisting mainly of aggregated beta-amyloid (Aβ) protein and intracellular neurofibrillary tangles (NFT) the main constituent of which is the cytoskeletal protein Tau (see Figure 1-1).



Tau based neurofibrillary tangle

Amyloid based Neuritic plaque



(c)

Figure 1-1: Detailed images of NFT (a) and extracellular plaques (b) seen in AD (from Munoz and Feldman 2000). (c) clearly illustrates the cellular localisation of these pathological characteristics (Cole 2006).

The Aβ present in plaques is produced from post-translational processing of the transmembrane protein amyloid precursor protein (APP). The normal physiological role of APP is unclear but it has been speculated to be neurotrophic and to have roles in development and memory as well as being involved in cell adhesion and cell signalling via intracellular protein interactions (Wolfe and Guenette 2007). Furthermore Aβ, derived from APP, may have a function as a defence molecule in normal ageing although it initiates toxicity when it is allowed to accumulate in AD (Campbell 2001). During amyloidogenic processing APP is

cleaved by β secretase to leave APPsβ as well as a C99 fragment which after γ secretase cleavage produces Aβ along with the APP intracellular domain (AICD) fragment (see Figure 1-2). Alternatively APP can undergo non-amyloidogenic processing by α secretase cleavage to produce APPsα and C83 fragments, the C83 fragment then undergoing γ secretase cleavage to produce P3 and AICD fragments (see Figure 1-2). In non-amyloidogenic processing α secretase cleaves APP in the centre of the Aβ peptide sequence and therefore prevents the production of Aβ (see Figure 1-2). The relative activity of α secretase compared to β secretase therefore has a crucial effect on the amount of Aβ that is produced.

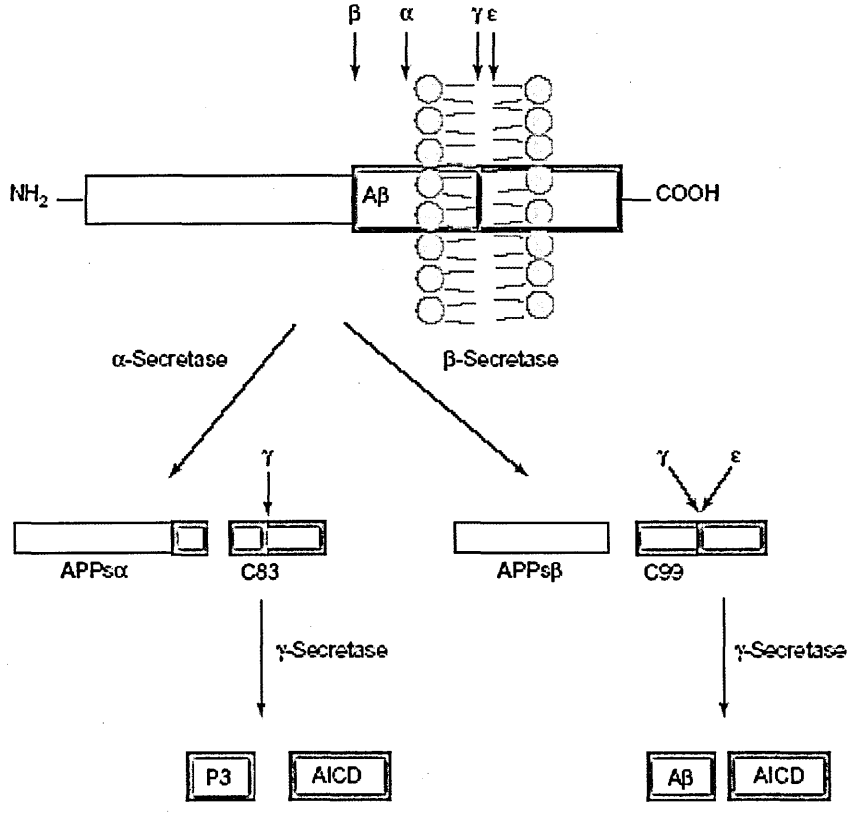


Figure 1-2: The amyloidogenic and non amyloidogenic processing of the transmembrane protein APP via α or β and then γ secretase activity. From LaFerla and Oddo (2005).

The two main forms of Aβ that are observed naturally are the 1-40 and 1-42 fragments and the secretases involved in APP processing and fragment formation have been identified. The aspartic protease, BACE , has been identified as having β-secretase activity (Vassar *et al.* 1999) and α-secretase activity has been attributed to a protein named ADAM-10 while γ secretase activity has been found to be due to a protein complex containing presenilins (PS) 1 and 2 and nicastrin (Pastorino and Lu 2006). The γ secretase complex is associated with APP cleavage products in the early secretory pathway implying that this pathway may be the focal point of amyloidogenic processing (Selivanova *et al.* 2007).

NFT tangles are formed from aggregates of the cytoskeletal protein Tau. It has been shown that the aggregation of Tau protein is caused by β-hyperphosphorylation of the protein and that this aggregation may lead to the subsequent formation of NFT and increased toxicity (Grundke-Iqbal *et al.* 1986; Gomez-Ramos *et al.* 2006).

Severe neuronal damage occurs in AD leading to lesions containing dystrophic neurites and activated glia throughout the central nervous system (CNS) (Price *et al.* 1998). These lesions affect large areas of the brain, including the limbic system which leads to impaired memory formation, and therefore cognitive decline occurs as described in section 1.1 (Hajilou and Done 2007). Despite the general atrophy that occurs in the CNS during AD there are CNS areas that are particularly susceptible to degeneration including the medial temporal lobe where severe thinning i.e. cell loss, not seen in normal ageing has been found in AD patients (Dickerson *et al.* 2009). The cholinergic system has also been found to be particularly vulnerable in AD. Cholinergic markers including acetylcholine (ACh) and choline acetyltransferase, which catalyses the formation of ACh from choline and acetyl-CoA, decrease in the frontal and temporal lobes of patients with AD (Bartus *et al.*

1982) as well as in transgenic mouse models of AD (Bellucci *et al.* 2006). Some of these changes have been demonstrated to occur in the hippocampus from middle age in normal ageing although this age-related decrease has been found to be more severe in AD patients (Perry *et al.* 1992) and there are studies that show little change in these markers in the elderly compared to the young (Bartus *et al.* 1982). The cholinergic system has been demonstrated to have a crucial role in learning and memory by human studies using anticholinergics as well as animal lesion experiments (Schliebs and Arendt 2006) and therefore these changes in the cholinergic system could be largely responsible for the initial deficit seen in patients in relatively early stages of AD. This theory is supported by the fact that these cholinergic deficits have been found using work on transgenic mouse models of AD to correlate with cognitive impairments at 7 months as demonstrated using the step-down inhibitory avoidance test of memory and learning (Bellucci *et al.* 2006). As well as these presynaptic changes, alterations in the muscarinic ACh receptors (mAChR) in the limbic system have been demonstrated (Rinne *et al.* 1985; Pakrasi *et al.* 2007). Moreover the nicotinic ACh receptors (nAChR) containing the α-7 subunit appear to be particularly implicated in leading to neurodegeneration as $A\beta_{1-42}$ has been demonstrated in neuroblastoma cell cultures to co-localise with these nicotinic acetylcholine receptors and to selectively accumulate in neurons with higher densities of these receptors (Nagele *et al.* 2002). Furthermore it has been shown in hippocampal cell cultures that Aβ can block the response of these nicotinic acetylcholine receptors in a non-competitive, voltage independent manor (Liu *et al.* 2001).

Although the cholinergic deficit is very severe and occurs early in the disease process (Lowe *et al.* 1988; Bell and Cuello 2006) other neurotransmitter systems are also affected in AD. Gamma Aminobutyric Aacid (GABA)ergic neurons have been

found to be relatively unaffected in the early stages of the disease but a decrease in the number of these neurons has been demonstrated in later stages of the disease in several areas including the temporal cortex (Lowe *et al.* 1988; Bell *et al.* 2006). Furthermore Paula-Lima *et al* (2005). demonstrated that activation of the $GABA_A$ receptors could prevent the toxicity of Aβ implying a crucial role for GABA inhibition in the disease process (Paula-Lima *et al.* 2005). This GABAergic deficit has been associated with AD symptoms such as depression in the later stages of the disease (Lanctot *et al.* 2007).

There is also extensive evidence for the loss of glutamate signalling in AD, both post-synaptic with internalisation and reduction of glutamate receptors (Lau and Zukin 2007; Parameshwaran *et al.* 2007) and degeneration of glutamatergic neurons, in particular affecting the hippocampal pyramidal neurons and therefore affecting memory formation (Francis 2003). Examination of AD brain specimens antemortem demonstrated decreased glutamate in the cortex and CSF in early disease patients and a loss of glutmatergic neurons as the disease progressed (Procter *et al.* 1988). Furthermore, reduced glutamate transporter levels have been observed in the parietal and occipital lobes of AD patients that correlated with the number of tangles in the temporal cortex (Kirvell *et al.* 2006).

1.1.2. Neurotoxicity in Alzheimer's disease

1.1.2.1. The primary cause of neurotoxicity

There is controversy over the primary cause of the neurodegeneration and lesions in AD. The current leading hypothesis is the amyloid hypothesis which states that it is the extracellular amyloid senile plaques that initiate neurodegeneration (Hardy and Higgins 1992) and that the NFT are a secondary effect caused by

changes in kinase activity (Pastorino and Lu 2006). This hypothesis has received support from experimental evidence showing that Aβ has a toxic effect leading to cell death in primary cell cultures including erythrocytes (Clementi *et al.* 2007), hippocampal cells (Alvarez *et al.* 2004) as well as numerous cell lines (Li *et al.* 1996; Costantini *et al.* 2005). Furthermore, Aβ in culture has been shown to cause the phosphorylation of Tau protein which can lead to its aggregation and therefore induce the assembly of NFT, and Tau has been found to be toxic to cells in culture in an aggregation-dependent manner (Gomez-Ramos *et al.* 2006). Aβ has also been found to directly influence Tau filament formation by inducing a caspase cascade which produces a toxic 17KDa fragment of Tau that may more easily aggregate (Gamblin *et al.* 2003). However, there is also a school of thought that says it is the Tau NFT formation that precedes the Aβ aggregation and neurodegeneration which is supported by evidence showing that phosphorylated Tau levels are a better indicator of disease severity than Aβ (Andersson *et al.* 2008) and that Tau has been found to be necessary for the toxicity of Aβ as Tau depleted neurons were not susceptible to Aβ-induced toxicity (Rapoport *et al.* 2002).

Inflammation occurs in AD and may be of considerable importance in contributing to the effects of AD (Munoz and Feldman 2000; Sastre *et al.* 2008). Speciale *et al.* (2007) found that AD patients had a decreasing number of circulating B lymphocyte cells depending on the severity of disease as well as increased levels of $CD8^{+}CD71^{+}$ cells and a significant decrease in interleukin 10 (IL-10) production after stimulation of peripheral blood mononuclear cells with $A\beta_{1\text{-}40}$.

1.1.2.2. Beta-amyloid-induced toxicity

$A\beta_{1-42}$ has been shown to aggregate more easily and therefore exhibit greater toxicity on cells in culture than $A\beta_{1-40}$ (Zou *et al.* 2003; Liu *et al.* 2005; Lim *et al.* 2007) and it has even been suggested that $A\beta_{1-40}$ may be protective against $A\beta_{1-42}$-induced neurotoxicity (Zou *et al.* 2003). The shorter artificial $A\beta_{25-35}$ fragment has been found to elicit increased toxicity compared to the two longer, naturally occurring fragments and investigation showed that the principal cause of the difference in toxicity was the exposure of a C terminal methionine in the $A\beta_{25-35}$ (Varadarajan *et al.* 2001).

The mechanism by which Aβ leads to cellular toxicity is not yet clear despite extensive research. However it is known that there is a strong involvement of several cell signalling pathways (see Figure 1-3). These may lead to the increased phosphorylation of Tau which causes the accumulation of NFT and consequently more toxicity. For example the cell cycle regulating transcription factor, p53, has been found to be increased in AD patients and *in vitro* in HEK293 cells and causes Tau phosphorylation (Hooper *et al.* 2008). Moreover the phosphorylation at Tyr216 and activation of the kinase, glycogen synthase kinase 3β (GSK3β) has been widely associated with the mechanism of the toxicity of Aβ by extensive evidence (Kaytor and Orr 2002; Liu et al. 2005; Hooper *et al.* 2008; Hu *et al.* 2008) and GSK3β inactivation via phosphorylation at Ser 9 has been shown to prevent Aβ-induced toxicity (Liu *et al.* 2005). GSK3β has been found to accumulate in NFT in the AD brain (Kaytor and Orr 2002) and in mice overexpression of GSK3β has been demonstrated to decrease long term potentiation (LTP) (Hooper *et al.* 2008). The effect of GSK3β on Aβ-induced toxicity may be mediated in several ways. The effect could be via phosphorylation of GSK3βs' substrates β-catenin and heat shock factor-1 which would decrease their ability to regulate gene expression (Kaytor and Orr

2002), directly via apoptosis, or via causing an increase in Aβ produced from APP by altering the activity of the β and γ secretase (Hooper *et al.* 2008). GSK3β has also been found to be essential to the Aβ-induced phosphorylation of Tau (Hu *et al.* 2008). Furthermore, the cyclin dependent kinase 5 (Cdk5) has been implicated in Aβ-induced toxicity because activity and stability of the Cdk5-p35 complex has been observed to increase in hippocampal neurons that have been treated with $A\beta_{1-40}$ *in vitro* (Alvarez *et al.* 2001).

Another cell signalling pathway that has been associated with Aβ-induced toxicity is the p75 neurotrophin receptor (NTR) cell death signal. Hippocampal injection of $A\beta_{1-42}$ has been shown to only decrease the number of basal forebrain ACh neurons in mice that were expressing the p75 NTR demonstrating the importance of this receptor, which is normally highly expressed by cholinergic neurons, in Aβ-induced toxicity (Sotthibundhu *et al.* 2008). Moreover, Costantini *et al.* (2005) found that toxicity induced by $A\beta_{1-42}$ fibrils was p75 NTR dependent although toxicity mediated by soluble oligomers was not and the p75 NTR appears to be protective against this latter type of toxicity in SH-SY5Y, human neuroblastoma, cells.

The APP protein itself may exacerbate the toxicity of Aβ. APP has been found to make neurons more susceptible to Aβ-induced toxicity (Sola Vigo *et al.* 2008) and APP reduces ε protein kinase C (PKC) in cell lines (Liron et al. 2007). PKC can inactivate GSK3β so therefore the ability of APP to reduce PKC may cause an increase in GSK3β activity. APP has also been found to increase $A\beta_{1-40}$ and $A\beta_{1-42}$ -induced toxicity in hippocampal cultures by binding to the APP protein binding sequence, Go (Sola Vigo *et al.* 2008).

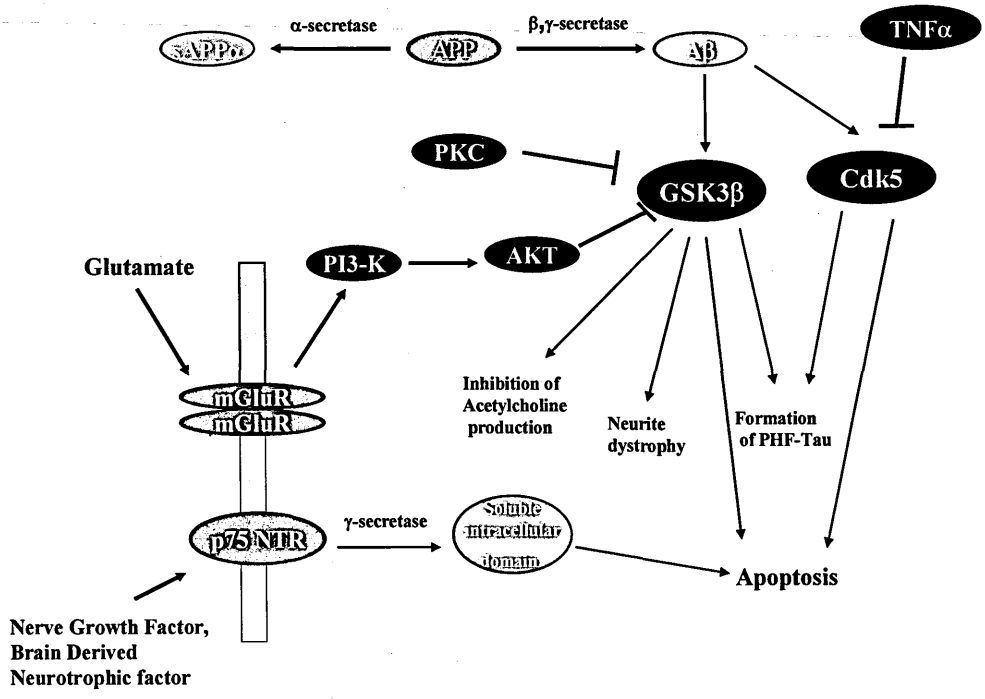


Figure 1-3: Some of the signalling pathways involved in the mechanism of Aβ-induced toxicity and their inhibitors. Modified from Klementiev *et al.* (2007).

1.1.3. Prevention of beta-amyloid-induced toxicity

1.1.3.1. Prevention of the aggregation of beta-amyloid

The importance of the aggregation state on the level of toxicity induced has been emphasised in Section 1.1.2.2. Aβ-induced toxicity may therefore be prevented by inhibiting the aggregation of Aβ. Trehalose (Liu *et al.* 2005) and a peptide derived from activity-dependant neuroprotective protein (ADNP), NAP, which binds to microtubules, have both been shown to be protective against Aβ-induced toxicity by preventing the aggregation of Aβ (Ashur-Fabian *et al.* 2003). The polyphenol group, stilbenes, have also been demonstrated to prevent the aggregation of $A\beta_{25-35}$ (Riviere *et al.* 2007). However, not all compounds that prevent Aβ aggregation are protective and α-antichymotrypsin was not found to be neuroprotective despite inhibiting the formation of fibrils (Aksenova *et al.* 1996).

1.1.3.2. Prevention of beta-amyloid-induced signalling

The detailed mechanism of Aβ-induced toxicity is not definitively known but several pathways have been implicated as was discussed in detail in Section 1.1.2.2. The protective effects of a compound may therefore act via the inhibition of one of these signalling cascades. The protective effects of the antioxidant, Trolox, and 17β oestradiol against $A\beta_{1-40}$-induced toxicity in hippocampal cultures have been attributed to preventing the Aβ-induced increase in activation of GSK3β via activation of the Wnt pathway (Quintanilla *et al.* 2005). Activation of metabotropic glutamate receptor 5 also confers protection against $A\beta_{1-40}$- induced toxicity in hippocampal slice cultures via activation of phosphoinositide-3 kinase (PI3K) and the serine/threonine kinase, Akt, which leads to inactivation of GSK3β (Liu 2005). α lipoic acid had also been demonstrated to exert protective effects against $A\beta_{31-35}$ via activation of the PI3K/Akt pathway. Moreover lithium chloride is protective against behavioural deficits in APP transgenic mice by alteration of the GSK3β pathway (Rockenstein *et al.* 2007).

The cytokine TNFα has been found to be protective against Aβ-induced toxicity in hippocampal cultures by decreasing Cdk5 protein levels and activity (Orellana *et al.* 2006) and by preventing Aβ-induced increases in ROS (Barger *et al.* 1995). This effect on ROS has been attributed to κB dependent transcription (Barger *et al.* 1995). However Stepanichev *et al.* (2006), found that TNFα prevented Aβ-induced cognitive impairment in rats *in vivo* but did not prevent Aβ-induced toxicity *in vitro*.

1.1.4. Risk factors for Alzheimer's disease

Age is the biggest risk factor for AD as discussed above in Section 1.1, with 50% of people over 85 developing the disease. The most common form of the disease has a sporadic onset without necessarily any family history although there are familial forms of AD (FAD) which have a genetic linkage and account for around 5-10% of AD cases (Maccioni *et al.* 2001). The age of symptomatic onset tends to be earlier in FAD than in sporadic AD and decreases through the generations that the disease is passed on. These FAD families have been widely studied in order to gain insight into the causes of AD. As may be expected given the role of APP processing and the aggregation of Aβ in AD initiation and progression, many of the genes that have been associated with AD are related to the processing of the APP protein. A link has been found between AD risk and several different mutations in the APP gene on chromosome 21 (Elbaz *et al.* 2007). Furthermore mutations in the genes encoding the γ complex proteins, PS1 and 2 on chromosomes 14 and 1 respectively have also been implicated in FAD (Wragg *et al.* 1996; Raman *et al.* 2007; Wu *et al.* 2007). PS1 in particular has been demonstrated to have a highly significant impact on the risk of AD not only in the early onset disease but also for late onset patients (Wragg *et al.* 1996).

Down's syndrome patients have an extremely high risk of developing AD due to the presence of an extra copy of chromosome 21 and therefore increased APP. Moreover as well as increased APP levels, low α secretase and high β secretase levels have been seen in Down's syndrome patients (Nistor *et al.* 2007).

Gender is also an important factor in disease progression with women being twice as likely as men to develop AD if factors such as age, level of education and physical activity are the same. This sex linkage may be due to increased average life span in women compared to men or may be related to hormonal changes in

menopausal women especially given the reported connection between oestrogens and brain derived neurotrophic factor (Baum 2005; Sohrabji and Lewis 2006).

One of the well-documented risk factors for AD is the apolipoprotein E (ApoE) allele which is expressed. The presence of the ApoE4 allele has been demonstrated to increase risk of late onset AD (Corder *et al.* 1993). In contrast the presence of the ApoE2 allele has been found to decrease the risk of developing AD (Corder *et al.* 1994; Elbaz *et al.* 2007) (see Figure 1-4).

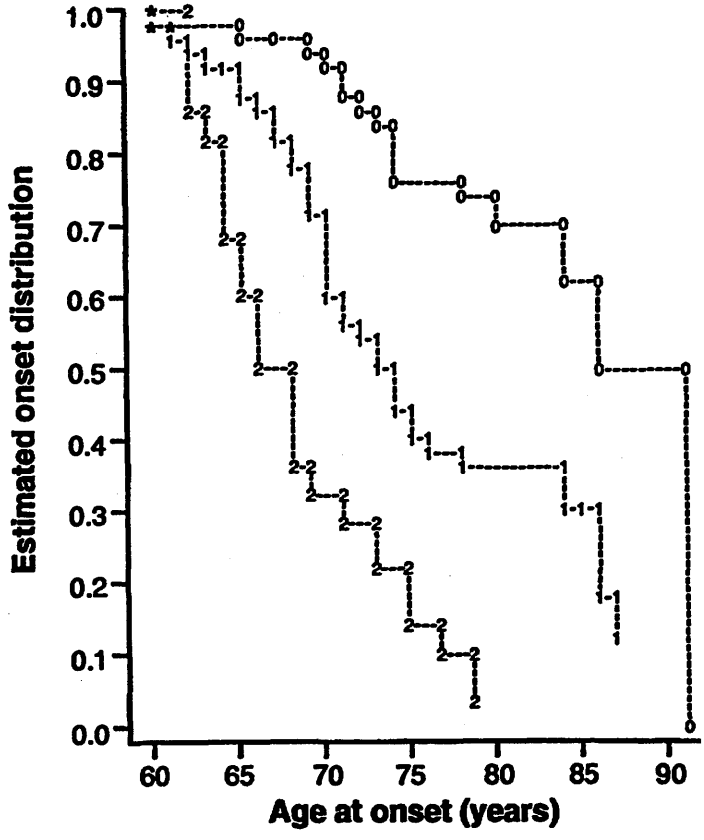


Figure 1-4: The effects of ApoE phenotype on AD risk.
This figure illustrates the link between ApoE phenotype and comparative risk of being diagnosed with AD. This shows the relative age of onset of AD for individuals with 0, 1 or 2 alleles of ApoE4. (From Corder *et al.* 1993).

The cause of this link between ApoE and AD is unclear but it may be due to oxidative stress in the AD brain as Montine *et al.* (1997) have shown that the lipid peroxidation product 4 hydroxynonenol (4HNE) differentially modifies the ApoE

protein (Montine *et al.* 1997). Alternatively as the ApoE receptor 2 has been demonstrated to be involved in neuronal survival in adults it has been suggested that the ApoE allele that is present may affect receptor activation and neuronal survival (Beffert *et al.* 2006).

As illustrated in Figure 1-5 as well as these genetic influences there are environmental factors that have been shown to affect the incidence of AD. Smoking has previously been reported to be beneficial in preventing the onset of AD (Hillier and Salib 1997) but this effect is unclear and recent studies have shown that smoking does not have any significant correlation with the development of AD (Doll *et al.* 2000; Tyas *et al.* 2000) or indeed that it can increase the risk (Juan *et al.* 2004; Reitz *et al.* 2007). What is clear is that there is a strong link between increased AD risk and high LDL or total cholesterol which in turn links AD risk to cardiovascular disease risk (Michikawa 2003). Furthermore there is evidence that life style, in terms of mental and physical activity, can alter risk of dementia as it has been shown that a more mentally challenging job could decrease risk of AD (Potter *et al.* 2008) and that physical activity has a positive effect in maintaining cognitive health (Jedrziewski *et al.* 2007). This potential benefits of anaerobic exercise on cognitive health may be related to neurogenesis in the hippocampus since running has previous been demonstrated to improve cell proliferation and neurogenesis in the mouse dentate gyrus (van Praag *et al.* 1999) and to enhance learning and hippocampal neurogenesis in aged mice (van Praag *et al.* 2005). There is also evidence that anaerobic exercise may lead to a compensatory increase in oxidative defences in order to cope with the increased oxygen consumption and this may therefore prevent oxidative damage which could contribute to disease pathology (Radak *et al.* 2008).

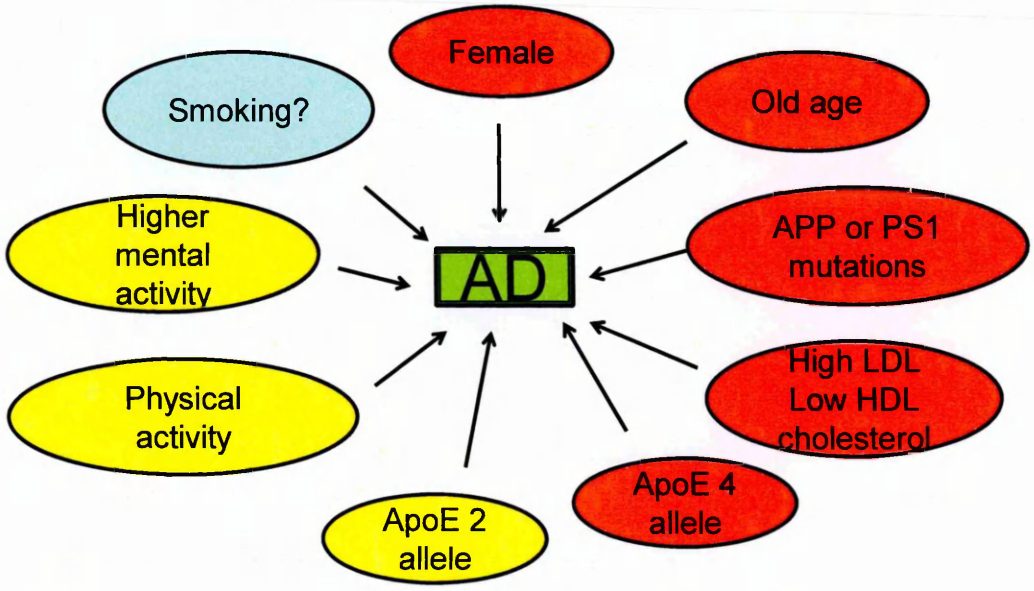


Figure 1-5: Schematic of the various influences on AD risk. Positive influences (leading to a decrease in risk of AD) are in yellow, negative influences (leading to an increase in risk of AD) are in red and those which have an uncertain effect are in blue.

There may be interactions between different risk factors, so for example the effect of the presence of the ApoE4 allele on hippocampal volume has been found to depend on gender (Fleisher *et al.* 2005) and ApoE phenotype is linked to cholesterol levels (Michikawa 2003). Moreover Down's syndrome patients already have a high risk of developing dementia but this has been seen to be further increased in those with higher blood cholesterol levels (Zigman *et al.* 2007).

1.1.5. Diagnosis

With any disease or illness early diagnosis is essential for the best possible outcome to the patient and in the case of AD it may mean being able to slow disease progression. The problem is that although diagnosis of AD is comparatively simple post-mortem when NFT and plaques mat be stained and observed in brain samples, it has been found to be difficult to conclusively diagnose AD as opposed to any other form of dementia as the definitive cause of dementia whilst the patient is alive. For example there is a remarkable symptomatic overlap between AD and vascular dementia, which is the second most common form of dementia, leading to loss of short term memory as well as sensory and motor disturbances and usually caused by ischaemic insult rather than any genetic influence (Yagami 2006). Nevertheless with the advent of improved technology alongside the traditional diagnostic cognitive tests including Alzheimers' Disease Assessment Scale (ADAScog) and the Mini Mental State Evaluation (MMSE) diagnosis is gradually becoming more definitive and more research is going on to develop biochemical diagnostic tests. For instance techniques now allow the levels of factors such $A\beta_{1-42}$ or Tau in the cerebrospinal fluid (CSF) to be used as markers of AD. However the procedure used to extract CSF is not straightforward and (Stefani *et al.* 2006) found that only Tau levels but not $A\beta_{1-42}$ correlated with cognitive decline.

Reasonable reliability for diagnosis has, however, been seen with blood markers such as oxidatively-modified peroxiredoxin. Yoshida *et al.* (2009) and Gruden *et al.* (2007) found that the levels of autoantibodies to $A\beta_{25-35}$, the astrocyte-specific protein S100b as well as the neurotransmitter dopamine, reflecting the loss of the dopaminergic system in AD, were good markers of disease progression. The amounts of the $A\beta_{1-40}$ and $A\beta_{1-42}$ forms in plasma and in particular the ratio of the two forms has quite recently been found to correlate with severity of disease and the

risk of conversion from mild cognitive impairment (MCI) to AD (Shoji 2002; Graff-Radford *et al.* 2007; Wiltfang *et al.* 2007). Furthermore the use of the techniques of magnetic resonance imaging and positron emission tomography as diagnostic tools has increased and can be useful for diagnosis even in very early stages of disease (Mosconi *et al.* 2007).

1.1.6. Present Alzheimer's disease treatments

The favoured treatment strategies for AD have varied over time and at present are not really satisfactory in the longer term. Currently the only effective treatment is to deal with the symptoms.

As the cholinergic system is especially vulnerable in AD some of the most common current treatments work on the acetylcholine system, in particular using cholinesterase inhibitors which prevent the breakdown of Ach and therefore help to maintain the cholinergic system and can afford symptomatic relief although mild side effects are common (Birks 2006). However, the gender of the patient has been found to alter the effectiveness of these drugs (Haywood and Mukaetova-Ladinska 2006) and their benefits may be related to preventing glutamate mediated toxicity (Takada-Takatori *et al.* 2006). Several cholinesterase inhibitors have been tested in clinical trials and reviews of these trials showed beneficial effects for rivastigmine (Birks 2006), Galantamine which has shown improvement in patient ADAS-cog scores as well as improved or stabilised global ratings (Loy and Schneider 2006) and Donepezil which has demosntrated beneficial effects on ADAS-cog, MMSE and global clinical state of patients with mild to moderate AD over 24 weeks (Birks and Harvey 2006).

The discrepancy in gender risk for AD, especially in post-menopausal women, implies a link between AD and oestrogen level changes and therefore the

potential of hormone replacement therapy (HRT) to reduce the incidence of AD has been investigated. The results of these studies have been variable. The use of HRT has been associated with a decreased AD risk in many studies (Zandi *et al.* 2002; Baum 2005) and oestradiol has been found to prevent memory deficits seen in rats treated with $A\beta_{1-42}$ (Hruska and Dohanich 2007) as well as interfering with the formation of Aβ fibrils (Morinaga *et al.* 2007). However the literature discussing the potential benefits of HRT as a preventative measure against AD is still rather unclear. Some studies have shown a positive correlation between HRT and AD with progesterone being considered detrimental (Honjo *et al.* 2005). Interestingly Honjo *et al.* (2005) found that a non-feminising oestrogen form, J861, can have the same benefits as oestradiol *in vitro* in PC12 cells as well as in the rat CNS (Honjo *et al.* 2005) and could potentially be a more practical long-term treatment for AD.

Antioxidant defence and the oxidative stress related damage that is found in AD patients is discussed in detail in Section 1.3.3. Another main area of research for AD therapies is that of compounds with antioxidant properties that will ameliorate this increase in oxidative stress related damage. For example vitamin E has been shown *in vitro* to prevent some of the effects of Aβ including increasing the activation of inducible NOS, which is present in the CNS and the cardiovascular system and responds to stress signalling, and ROS production *in vitro* (Ayasolla *et al.* 2004; Esposito *et al.* 2006). Furthermore the antioxidant effects of blueberry extract have been investigated and it was found to be neuroprotective in kainic acid treated rat hippocampi (Duffy *et al.* 2008). However, a review conducted by (Isaac *et al.* 2008) found that there was no evidence of the efficacy of vitamin E to treat AD or MCI.

Drugs to reduce cholesterol have been tried as therapeutics, because high circulating levels of cholesterol has been associated with greater susceptibility to

development of AD. These trials have shown positive results with ADScog scores significantly better in AD patients on the cholesterol lowering drug atorvastatin than controls (Sparks *et al.* 2006). This treatment has also been found to reduce the AD risk in Down's syndrome patients with high cholesterol (Zigman *et al.* 2007). Moreover the effects of cholesterol-lowering drugs have been studied in rats and it was found that the drugs which could cross the blood brain barrier were acting as AChE inhibitors and therefore would be particularly beneficial as therapeutics (Cibickova *et al.* 2007). However, despite these promising reports a review of the clinical data suggests that statins do not give clinical benefits to preventing AD (Scott and Laake 2001).

Given the importance of Aβ and its aggregation state for the toxicity of the peptide and the development of AD, compounds which decrease Aβ load and prevent aggregation or cause disaggregation are of interest from a therapeutic viewpoint. To this end Arendash *et al.* (2006) studied the effects of caffeine and found that it could decrease Aβ production, and antioxidants such as polyphenols and vitamins E and C not only reduce the oxidative stress burden in the AD brain but also in some cases prevent Aβ aggregation (Ono *et al.* 2006). There is also research into the use of secretase inhibitors to reduce the production of Aβ (Lecanu and Papadopoulos 2007).

The idea of an AD vaccine has been explored in order to prevent excess Aβ accumulation and aggregation and therefore prevent disease formation. There have been promising results from some studies. Antibodies against Aβ have been shown to reduce intracellular Aβ *in vitro* in neuroblastoma cells and primary neurons (Tampellini *et al.* 2007). Vaccination of APPswe/PSI transgenic mice with a $A\beta_{1-42}$ gene construct led to antibody production against $A\beta_{1-42}$ and decreased $A\beta_{1-42}$ levels in the brain and reduced hippocampal plaques by 52% (Qu *et al.* 2007). Kim *et al.* (2007) found that they could elicit more reliable antibody responses in mice by using

immunisation with a vector and DNA plasmid containing tandem repeats of $A\beta_{1-6}$. Passive immunisation of mice with single chain antibodies against Aβ have been demonstrated to inhibit Aβ aggregation and to decrease plaque burden in the brain when injected into the hippocampus or intramuscularly (Wang *et al.* 2009). Furthermore, it has been found that active vaccination of human patients with AN-1792 caused an antibody response and may reduce the plaque burden in the CNS in human patients (Nicoll *et al.* 2003). However, the active vaccination does not appear to alter the levels of NFT unless given very early (Nicoll *et al.* 2003) and may not have large effects on cognitive function (Gilman *et al.* 2005; Wisniewski and Konietzko 2008) and the safety of active vaccines was called into question when a clinical trial found that some patients, 6%, developed an inflammatory response and meningoencephalitis (Nicoll *et al.* 2003; Gilman *et al.* 2005). Passive immunisation has been shown to have potential efficacy on cognitive function in patients without the ApoE4 allele and will not cause the inflammatory response seen with active vaccines so there are several phase I and II clinical trials presently underway or being concluded (Wisniewski and Konietzko 2008). More novel approaches to vaccination are also being investigated and recently Cao *et al.* (2008) have investigated the possibility of vaccines using sensitized, bone marrow derived, dendritic cells. The authors found that in BALB/c mice there was a strong antibody response when dendritic cells were sensitized with mutated $A\beta_{1-42}$ but not when sensitized with wild type $A\beta_{1-42}$ although an anti-inflammatory immune Th2 response was observed independent of the form of $A\beta_{1-42}$ that had been used (Cao *et al.* 2008).

In addition to the potential benefits of physical and mental exercise, HRT and antioxidants, it has been shown that a diet rich in arachidonic and docosahexaenoic fatty acids may be preventative against the onset of AD symptoms (Kotani *et al.* 2006). However a review of studies on fatty acids and dementia concluded that there

was a not a preventative benefit of using fatty acids against dementia (Lim *et al.* 2006).

1.1.7. Animal models of Alzheimer's disease

Many mouse models of AD are available for use in research studies. These transgenic mice may have a mutation in the APP gene, the PS1 gene, the gene for Tau or a combination of these mutations and the specific characteristics of these models vary depending on the mutation used. The most commonly used APP mutation is the Swedish mutation, first found in a family with AD in Sweden at residues 670/671 which affects β secretase activity and mice with this mutation have been shown to have impaired reversal learning at 6 months with increasing prefrontal cortex levels of $A\beta_{1-42}$ (Zhuo *et al.* 2008) as well as altered novelty seeking prior to plaque formation (Senechal *et al.* 2008). Double transgenic animals are more commonly used as they employ APP mutations with PS1 or Tau mutation in order to display more of the phenotypic characteristics of AD. APP/Tau transgenic mice for example have been found to have increased Aβ deposition and phosphorylated Tau accumulation in the brain leading to tangles as well as plaques and neurodegeneration with neuritis resembling those types seen in AD brains (Paulson *et al.* 2008; Perez *et al.* 2008). APP/PS1 transgenic mice have been demonstrated to have increased apoptosis in the cortex and hippocampus compared to wild type mice (Yang *et al.* 2008), decreased proliferation in the subgranular zone (Niidome *et al.* 2008) and deficits in both passive avoidance and left-right discrimination learning (Filali *et al.* 2008). Some transgenic mice include mutations in all three proteins, APP, Tau and PS1 and these have been found to develop increased intracellular Aβ, Tau accumulation then extracellular Aβ which seems to correspond to cognitive deficits seen at around 6 months and decreased neurogenesis and finally NFT

(Mastrangelo and Bowers 2008; Pietropaolo *et al.* 2008; Rodriguez *et al.* 2008). Triple transgenic mice have also been demonstrated to have synaptic deficits as well as impaired learning and memory (Cole 2006). Although these transgenic animals acquire plaque formation and Tau fibrils and show evidence of cognitive deficits they do not usually display the psychological effects of AD such as depression, although the APP/PS1 transgenic mice have been considered by Filali *et al*, (2008) to be demonstrating signs of apathy and depression by increased irritability, poor nest building and immobility in forced swimming tests.

1.2. Mechanisms of cellular death *in vivo* and *in vitro*

1.2.1. Apoptosis and necrosis

There are two main types of cell death that are observed in cells *in vitro* and these are referred to as apoptosis and necrosis. Apoptosis or programmed cell death is an ordered, caspase-dependent process and is characterised by the activation of apoptotic cascade molecules, DNA fragmentation, chromosome condensation and membrane potential changes (Yan and Shi 2005; Okouchi *et al.* 2007). Apoptosis occurs when cells are programmed to die during development, as a result of cellular stress and certain disease conditions such as Aβ-induced cell death in AD (Forloni *et al.* 1993). Necrosis is considered a much less ordered form of cell death because it is caspase -independent and involves cell lysis with the release of cellular contents (Okouchi *et al.* 2007) but does not involve the complicated signalling cascades that are involved in the induction of apoptosis. The triggers for cell death via apoptosis differ from those for necrosis with necrosis being initiated via mitochondrial permeability defects and a consequent reduction in ATP production or excess

reactive oxygen species (ROS) (Bhatia 2004). However it has more recently become clear that necrotic cell death can be more regulated than was originally thought and there are pathways for the initiation of necrosis (Proskuryakov *et al.* 2002; Xu *et al.* 2006; Okouchi *et al.* 2007). For example it has been demonstrated that death via poly (ADP-ribose) polymerase -1 is a form of regulated necrosis (Xu *et al.* 2006). The distinction between types of cell death is therefore not as clear as was initially thought.

1.2.2. Mechanisms of apoptosis

There are several mechanisms by which a cell may be assigned for apoptosis and once ordered the exact pathway by which apoptosis occurs may differ depending on cell type and the initiating factor. Some of the signalling pathways that are involved in this process are shown in outline form in Figure 1-6. Apoptosis can be initiated by molecules binding death signalling domain receptors on the cell surface or by toxic compounds or environmental effects such as radiation causing the release of cytochrome C from the mitochondria and either of these mechanisms will then cause a signalling cascade involving caspases (Yan and Shi 2005; Okouchi *et al.* 2007) (see Figure 1-6). Caspases are proteases that are activated when they are cleaved and then reform into active heterodimers. The caspase cascade is set in motion by the cleavage and activation of initiator caspases including caspase 8 and 9 which can subsequently cleave and activate the activator caspases such as caspase 3 and 7 (Yan and Shi 2005). The caspase cascade in turn sets in motion a chain of kinase activation that eventually leads to changes in gene expression switching on the expression of protease and DNA degrading enzymes leading to cell death. The intracellular pathways involved in apoptosis are complex; a large number of other

molecules have pro or antiapoptotic actions. The details of these pathways will not be discussed in detail here.

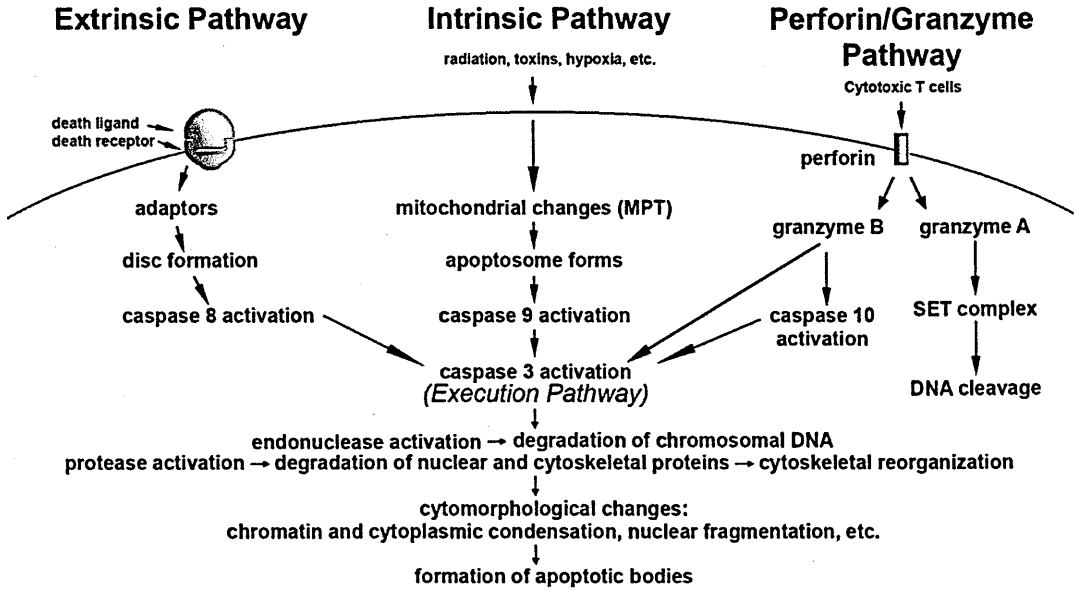


Figure 1-6: Mechanisms of the induction of apoptosis. This figure illustrates the mechanisms of the induction of apoptosis via death receptor-ligand interaction and mitochondrial disruption. From a review by Susan Elmore (Elmore 2007).

1.2.3. Apoptosis in beta-amyloid induced toxicity

There is evidence that apoptotic cell death and mitochondrial disruption are involved in the Aβ induced toxicity in different cell types. $A\beta_{25-35}$ has been shown to induce apoptosis in the SH-SY5Y cell line and both $A\beta_{25-35}$ and $A\beta_{1-40}$ have been found to induce apoptosis in hippocampal cultures (Forloni *et al.* 1993; Li *et al.* 1996). Furthermore in erythrocytes $A\beta_{1-42}$ has been observed to cause an increase in activated caspase 3 and to alter oxygen metabolism (Clementi *et al.* 2007). Hippocampal neurons have been demonstrated to be more susceptible to apoptosis in response to $A\beta_{1-40}$ or $A\beta_{25-35}$-induced increases in activated caspase 3 than cortical neurons and $A\beta_{25-35}$ has been found to disrupt Ca^{2+} homeostasis in these hippocampal neurons (Resende *et al.* 2007). $A\beta_{25-35}$ has also been seen to lead to decreased levels

of the anti-apoptotic molecule, Bcl-w, and increased levels of the pro-apoptotic, Bim, via JNK leading to exacerbated cytochrome C and Smac release from the mitochondria (Yao *et al.* 2007). However, despite the involvement of the initiation of apoptosis in Aβ-induced toxicity increased expression of the anti-apoptotic molecule Bcl-2 did not protect against $A\beta_{25-35}$ induced toxicity and cell death (Behl *et al.* 1993).

As well as increased caspase activation and evidence of apoptosis, mitochondrial dysfunction has been seen in relation to Aβ induced toxicity. For example in rat astrocytes Aβ has been found to cause an increase in phospholipase A2 via the activation of MAPK and NADPH leading to increased ROS and mitochondrial dysfunction (Zhu *et al.* 2006; Wang *et al.* 2007). Furthermore, Aβ has been demonstrated to cause Ca^{2+} increase in astrocytes leading to mitochondrial dysfunction (Abramov and Duchen 2005).

1.2.4. Prevention of beta-amyloid-induced initiation of apoptosis

Aβ leads to increased apoptosis (Forloni *et al.* 1993; Li *et al.* 1996; Awasthi *et al.* 2005) and mitochondrial dysfunction (Zhu *et al.* 2006; Wang *et al.* 2007) as detailed in Section 1.3.3 and compounds may protect against Aβ-induced toxicity by preventing this Aβ-mediated induction of apoptosis. For example oestrogen has been found to protect hippocampal cultures from $A\beta_{1-40}$-induced toxicity by preventing Aβ-induced activation of the apoptosis mediator caspase 3 (Park *et al.* 2007). Oestrogen has also been demonstrated to reverse the $A\beta_{35-35}$-mediated down-regulation of the anti-apoptotic molecule, Bcl-2, and up-regulation of the pro-apoptotic molecule, Bcl-w, by preventing $A\beta_{25-35}$ activation of JNK (Yao *et al.* 2007). Furthermore the natural flavenoid derivative, icaritin, which has been shown

to have antioxidant and oestrogenic activity also protected rat cortical cultures against $A\beta_{25-35}$-induced toxicity and corrected the $A\beta_{25-35}$-mediated Bcl-2 imbalance via an oestrogen receptor-dependent pathway (Wang *et al.* 2007). The natural plant extract *Lycium barbarum* also protected rat cortical neurons by eliminating $A\beta_{1-42}$ and $A\beta_{25-35}$-induced increases in JNK and caspase 3 activation (Yu *et al.* 2005) and melatonin decreased $A\beta_{1-42}$ and $A\beta_{25-35}$-induced apoptosis in astroglioma cells (Feng and Zhang 2004).

1.2.5. Apoptotic markers

The study of apoptosis or the prevention of cell death as assessed by the level of apoptosis are crucial in the study of neurodegenerative disease and therefore reliable markers are needed for this process as a research tool. A commonly used marker of cell death *in vitro* is propidium iodide which is taken into cells which have compromised membranes and shows up as a fluorescent marker that may be seen under the fluorescent microscope (Zhang *et al.* 2007). However, demonstrating whether cell death is apoptotic or necrotic is not necessarily simple. *In vitro* several markers are used as signs that apoptosis has been occurring for cell death or protection experiments. These include demonstration of activation of the caspases, in particular the executioner caspase, caspase 3 (see Figure 1-6). Activation of this caspase may be seen using Western blotting and antibodies specific to the activated form of the enzyme consisting of 12 and 17KDa units (Chiou *et al.* 2003; Ibuki *et al.* 2006) or using fluorescence activated cell sorting (FACS) analysis. The later apoptotic marker annexin V is also used to confirm the occurrence of apoptosis (Chiou *et al.* 2003; Zhang *et al.* 2007). DNA fragmentation is also used as a marker of apoptosis using nuclear markers such as Hoechst that will allow one to see DNA condensation by microscopy or by using electrophoresis (Ibuki *et al.* 2006). One of

the most commonly used assays to measure apoptotic activity though is Terminal deoxynucleotidyl Transferase (TdT) mediated dUTP nick end (TUNEL) labelling which detects DNA fragmentation (Thakur *et al.* 2007).

1.3. Oxidative stress

Oxidative stress refers to the physiological stress due to the presence of ROS such as the peroxide ($OH^{\bullet}$) and superoxide ($O^{\bullet -}$) radicals or reactive nitrogen species. These reactive species have an unpaired electron due to their involvement in reducing cellular reactions. The enzymes catalysing these reactions and the cycles of ROS production that may occur as a result of these reactions are shown in Figure 1-7. The unpaired electron makes these species highly reactive and they can oxidise proteins, lipids or even DNA causing cellular damage and releasing products such as 4-HNE, the aldehyde product of lipid peroxidation which is increased during oxidative stress, that can lead to more damage and eventually lead to cell death. This oxidative stress-related damage occurs when the balance between the cell's antioxidant defences and ROS that cause damage is shifted in favour of the ROS due to ageing, disease or mitochondrial dysfunction.

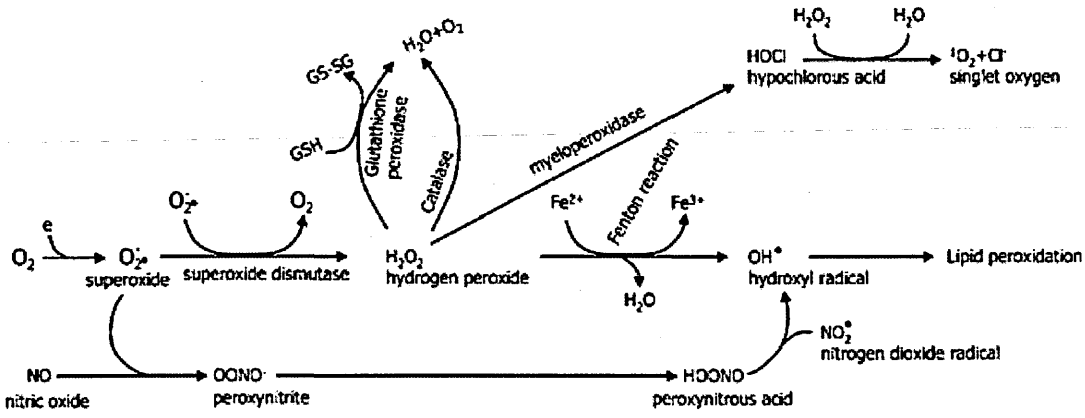


Figure 1-7: ROS production. The enzymatic reactions involved in ROS production. From (Chauhan and Chauhan 2006).

1.3.1. Antioxidant defence

The human body has extensive defence mechanisms in place to keep the redox balance in check and prevent oxidative stress-related damage. These defences include antioxidant enzymes to neutralise these ROS as well as non-enzymatic proteins and compounds that have antioxidant properties. The antioxidant enzymes include SOD which dismutates superoxide into oxygen and hydrogen peroxide (H_2O_2) and catalase which removes the resulting H_2O_2. Three forms of SOD occur naturally and these vary according to the metal ions that they require and their cellular localisation; SOD1 is cytosolic and requires Cu/Zn, SOD2 is localised to the mitochondrial matrix and contains Mn and SOD3 is an extracellular Cu/Zn SOD; with SOD1 being by far the most prevalent (Noor *et al.* 2002). As well as the anti-oxidant enzymes the compound glutathione produced by the enzyme glutathione synthetase has been shown to be crucial to antioxidant defence and this compound may be regulated in part by the anti-apoptotic Bcl-2 (Zimmermann *et al.* 2007). The vitamin E and C complexes have also been repeatedly demonstrated to have very strong antioxidant properties (Saeed *et al.* 2007; Traber and Atkinson 2007) and indeed Traber and Atkinson in 2007 argued in their review that the main function of

vitamin E is as a peroxyl scavenger (Traber and Atkinson 2007). Moreover a bioluminescence substrate, coelenterazine, has been found to have anti-oxidant effects and to be able to protect rat hepatocytes from H_2O_2 induced damage although the dose chosen is critical as coelenterazine itself has also been found to be toxic at certain concentrations (Dubuisson *et al.* 2000). Aside from having a healthy diet rich in vitamin antioxidants it has been shown that anti-oxidant defences can be increased with regular exercise, which causes adaptation to the anaerobic conditions as discussed in a review by Radak *et al.* (2008).

1.3.2. Oxidative stress in pathology

Oxidative stress has been considered to be at the root of many pathologies. Oxidative stress damage naturally accumulates throughout life and is widely thought to contribute to normal ageing as well as neurodegenerative diseases which are considered to be diseases of old age. In ageing it been reported that there are reductions in antioxidants which may contribute to memory decline as well as an increase in lipid peroxidation (Droge and Schipper 2007). The brain has low anti-oxidant defences and high oxygen consumption and as a consequence it is vulnerable to oxidative stress related damage. It is no surprise therefore that oxidative stress has been shown to have a role in almost all of the neurodegenerative diseases (reviewed in Reynolds 2007) with the presence of markers of oxidative stress damage in AD and PD and high ROS levels in MS (Noor *et al.* 2002; Bohr *et al.* 2007; Sayre *et al.* 2008).

Glial cells are also vulnerable to oxidative stress although astrocytes appear to be less vulnerable than oligodendrocytes and microglia (Hollensworth *et al.* 2000). Oligodendrocytes are particularly susceptible to oxidative stress and in culture have been demonstrated to release the receptor for advanced glycation end products,

which is involved in the activation of inflammatory genes at low levels of H_2O_2 and oligodendrocyte cell death is induced at higher H_2O_2 concentrations (Qin *et al.* 2008).

1.3.3. Oxidative stress in Alzheimer's disease

In AD it has become clear that whatever the exact mechanism of disease initiation there is an important role for oxidative stress-related damage in AD pathology and disease progression *in vivo* as well as in Aβ induced toxicity *in vitro*. The evidence for the involvement of oxidative stress in AD includes the observed increase in markers of oxidative stress including lipid and protein oxidation in patients with AD (Smith *et al.* 2000; Butterfield *et al.* 2001; Chauhan and Chauhan 2006) . Specifically the lipid peroxidation marker 4-HNE has been demonstrated to be increased in the brains of AD patients compared to age-matched controls (Williams *et al.* 2006) and oxidative adducts have been found in forms of the oxidative marker 14-3-3 in AD patients (Santpere *et al.* 2007).

The levels of SOD1 have been found to be increased and oxidatively modified in AD patients and furthermore this excess SOD1 was seen to occur in aggregates associated with plaques (Choi *et al.* 2005). The activity of SOD1 has also been demonstrated to increase in AD patients in several studies (Noor *et al.* 2002) including one by Serra *et al*, 1994, which showed increased SOD1 activity in red blood cell homogenate from AD patients compared to controls (Serra *et al.* 1994). The mRNA levels of SOD1 has been found to be unchanged in the CSF in AD patients but SOD1 was higher in AD patient skin fibroblasts than controls (Urakami *et al.* 1995). This oxidative stress may be directly linked to AD pathology as it correlates with the activity of the β-secretase molecule BACE1 (Borghi *et al.* 2006).

Changes in antioxidant enzymes have also been observed in animal models of AD. It has been observed that 4-HNE activity increases and SOD1 activity decreases in APP transgenic mice compared to controls (Schuessel *et al.* 2005). However, SOD1 and haemoxygenase 1 proteins increase in an APP Swedish mutation transgenic mouse model of AD (described in Section 1.1.7) and, as in human patients, the increase occurs mainly around Aβ deposits (Pappolla *et al.* 1998). The levels of Aβ plaques have been found to be increased in the brains of mice with partial knock out of SOD2 (Li *et al.* 2004) and SOD2 has been shown to be nitrated although protein levels were unchanged in PS1/APP transgenic mice (Anantharaman *et al.* 2006).

There is evidence *in vitro* that Aβ leads to oxidative stress and related damage. Harris *et al.* (1995), showed that $A\beta_{1-40}$ caused an increase in ROS and carbonyl oxidation but decreased gluatamine synthetase activity in hippocampal cultures from embryonic rats (Harris *et al.* 1995). Furthermore $A\beta_{25-35}$ has been found to cause lipid peroxidation in neuronal cultures from embryonic rats (Pike *et al.* 1997) and in synaptosomes from adult rats (Montiel *et al.* 2006). In hippocampal neurons in culture $A\beta_{1-40}$ has been demonstrated to increase SOD2 expression and temporally decrease SOD1 expression (Aksenov *et al.* 1998). However, Pappolla *et al*, (1998), saw an increase in SOD1 in PC12 cells derived from pheochromocytoma of the rat adrenal medulla that were treated with Aβ (Pappolla *et al.* 2003).

1.3.4. Anti-oxidant effects against beta-amyloid–induced toxicity

Oxidative stress has been directly implicated in AD and in Aβ-mediated cellular toxicity as detailed in Section 1.3.3 and several compounds that have been demonstrated to have antioxidant properties have been shown to protect against Aβ-induced toxicity. In particular vitamin E has been found to protect against Aβ-

induced memory deficits *in vivo* (Yamada et al. 1999). *In vitro* vitamin E has been shown to protect neurons against Aβ-induced damage (Montiel *et al.* 2006) and prevent the Aβ-mediated increases in ROS and iNOS expression (Ayasolla *et al.* 2004) . 17β-oestradiol has also been demonstrated to prevent the Aβ-induced oxidative stress mediated activation of p38 in MCF-7 cells in culture (Valles et al. 2008). The mechanism of this antioxidant effect of 17β-oestradiol has been suggested to be via activation of ERK1 and ERK2 which in turn causes translocation of NFκB, which up-regulates SOD2 expression (Borras *et al.* 2005). However, Pike *et al.* (1997) found that the antioxidant, probucol did not protect hippocampal cells from $A\beta_{1-42}$-induced toxicity (Pike *et al.* 1997).

1.4. The Limbic system and memory formation mechanisms

1.4.1. Limbic system structure and function

Because it is a key area affected in AD it is important to discuss the limbic system in some detail. The limbic system is a collection of brain areas situated primarily in the temporal lobe of the brain which together have been shown to be involved in memory consolidation, visceral motor activity and emotional responses to stimuli (Purves *et al.* 2001).

The limbic system is now generally accepted to include the cinglulate cortex, amygdala, hippocampus, thalamus, hypothalamus, cingulate gyrus and parahippocampal gyrus as well as some orbital and medial prefrontal cotical areas (Isaacson 1974; Purves *et al.* 2001). Whereas the hippocampus appears to only be important in contextual fear the amygdala has connections to and from the prefrontal cortex as well as the thalamus and has been shown to be critical for the emotional

responses to stimuli, in particular being demonstrated to be involved in conditional fear responses and anxiety (Zigmond *et al.* 1999; Purves *et al.* 2001). Some of these responses, including the release of stress hormones, emotional behaviour and reflex actions, are mediated by connections to the hypothalamus, via the striata terminalis and the ventral amygdalofugal pathways, and the reticular formation in the brainstem (Isaacson 1974; Zigmond *et al.* 1999). Connections from the limbic system area to the midbrain may also be important for the control that the limbic system shows via the activity of the reticular formation (Isaacson 1974).

This system and it's effects on motor responses is critical for shaping our behaviour in terms of recognising and remembering stimuli that we should be frightened of and therefore avoid. Furthermore this recognition also has clear advantages in that it allows us to recognise those who are unfamiliar and therefore may pose a threat.

1.4.2. The Hippocampus

1.4.2.1. Hippocampal structure and organisation

The hippocampus is part of the limbic system located in the temporal lobe of the brain and is known to have a major role in memory formation and the storage of long term memories via the processes of LTP and long term depression (LTD).

The cortex of the hippocampus is called archicortex as it contains only three or four cellular layers rather than the full six layers seen in the neocortex. The hippocampus consists of the CA1, CA2, CA3 regions and the dentate gyrus. The dentate gyrus receives inputs from the entorrhinal cortex and feeds connections via excitatory glutamatergic granule cells and mossy fibres onto the pyramidal cells of the CA3 region of the hippocampus. These pyramidal cells in the CA3 area extend

projections via the Shaffer collaterals to hippocampal areas CA1 and CA2 (see Figure 1-8). The glutamatergic pyramidal cells of the CA1 are the main output of the hippocampus having connections to the subiculum which then has connections to other parts of the limbic system including the amygdala and also the entorrhinal cortex. The excitatory outputs of the pyramidal neurons of the hippocampus are regulated by the activity of inhibitory outputs from a diverse population of GABAergic interneurons including the basket cells which surround the pyramidal axons and chandelier cells. As well as the excitatory and inhibitory neurons there are astrocytes, oligodendrocytes and microglia present in the hippocampus.

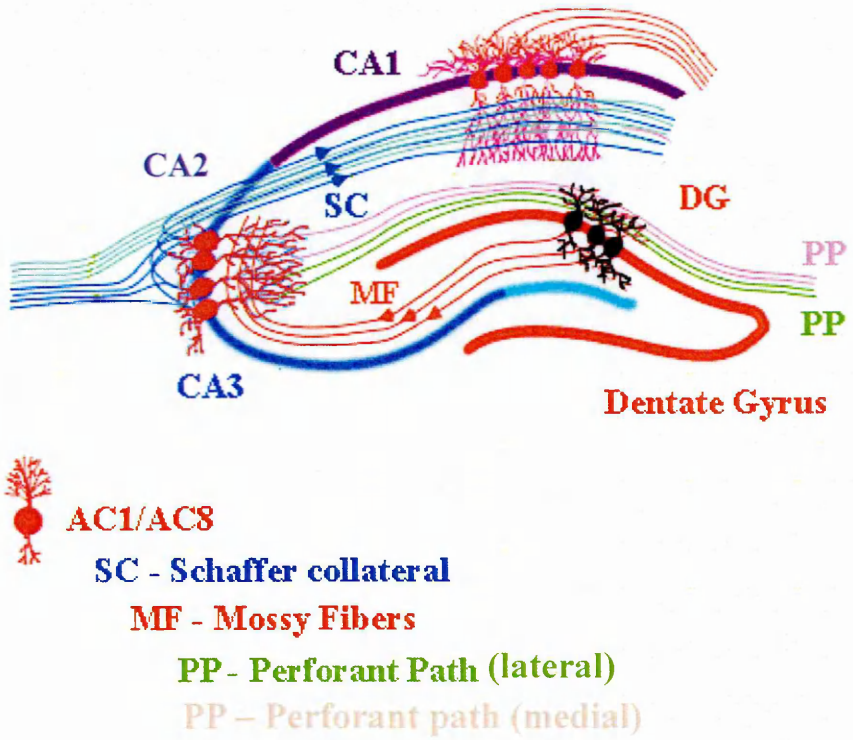


Figure 1-8: The circuitry of the hippocampus. Adapted from (Wang and Storm 2005).

1.4.2.2. The role of the hippocampus in long term potentiation and memory formation

LTP was first described by (Bliss and Lomo 1973). LTP refers to an increase in synaptic activity that leads to strengthening of synaptic connections between

specific neurons and in this way ultimately gives the foundations for memory formation (Bliss and Lomo 1973). LTP initiation involves the activation of NMDA glutamate receptors by removal of the magnesium block which usually exists in these receptors and this allows increased Ca^{2+} influx. The resulting Ca^{2+} influx can then initiate kinase cascades which eventually lead to downstream changes in gene expression via the transcription regulator CREB in the early stages of LTP. There is also increased glutamate AMPA receptor translocation to the post-synaptic terminal as a result of this cascade and even eventually new synapses may form. In this manner further increases in synaptic transmission occur and the synaptic strength increases still more (Bliss and Lomo 1973).

1.4.2.3. The rat hippocampus

The position of the rat hippocampus differs from that of the human hippocampus which lies in the temporal lobe. In the rat the hippocampus is higher up in the forebrain. *In vivo* the structure, cell types, efferent connections and function of the hippocampus have been shown to be very similar as for the human hippocampus as described in Section 1.4.2.1 with exceptions in that humans have a much larger CA1 area and have connections onto the association cortices which the rat lacks but the rat has much stronger commissural connections onto the dentate gyrus than the human (Anderson *et al.* 2006).

In vitro dissociated rat hippocampal cultures contain a diverse neuronal population, most of which are microtubule associated protein (MAP)-2 positive (Domenici *et al.* 2002). Glial fibrillary acidic protein (GFAP) is a marker of mature astrocytes and the number of GFAP positive cells has been seen to increase through development (Gasser and Hatten 1990). It has been found that only around 5% of the cells at E16-E18 are GFAP positive and this proportion increases to around 15% by

E20 (Gasser and Hatten 1990) but hippocampal cultures from both embryonic and early postnatal rats have also been demonstrated to contain GFAP positive astrocytes (Gasser and Hatten 1990; Domenici *et al.* 2002; Yamamoto *et al.* 2005; Hein *et al.* 2008). Moreover, O4 positive oligodendrocytes have been demonstrated to be present in hippocampal cultures (Xie *et al.* 2000; Hein *et al.* 2008). However Xie *et al.* (2000), did not find OX-42 positive microglia in hippocampal cultures from embryonic day 10 (E18) rats (Xie *et al.* 2000).

1.4.2.4. Hippocampus and Alzheimer's disease

As mentioned earlier the medial temporal lobe is highly vulnerable to the neurodegeneration seen in AD and the hippocampus in particular is one of the first areas to be affected. Hippocampal volume has been demonstrated to correlate with cognitive decline (Wolf *et al.* 2001) and MRI of people, with or without dementia symptoms, from families with a history of AD showed that hippocampal volume was reduced by as much as 25% in those with dementia compared to those without dementia symptoms (Wolf *et al.* 2008). Furthermore, the extent of hippocampal and entorrhinal atrophy have been found to be predictors of whether mild cognitive impairment will progress to AD (Devanand *et al.* 2007). The fact that these areas which are involved in memory formation and emotion are vulnerable and tend to be affected fairly early on in the disease process may explain some of the effects on memory.

The susceptibility of the hippocampus in AD related degeneration compared to other parts of the cortex has been attributed to differences in Ca^{2+} homeostasis due to the high levels of excitatory synapses present and the glutamate receptor subunits that they express (Brorson *et al.* 1995; Resende *et al.* 2007). Moreover some of the mutations associated with FAD have been demonstrated to affect the natural levels of

neurogenesis within the hippocampus which would also increase vulnerability as lost neurons could be replaced less easily (Verret *et al.* 2007).

1.5. Colostrinin

1.5.1. Colostrinin isolation, structure and properties

CLN is a uniform mixture of low molecular weight polypeptides consisting of around 30 constituent peptides of varying size with the smallest of these being 6KDa (Janusz *et al.* 1974). The whole CLN complex is 18KDa and was first isolated from ovine colostrum in early milk. The levels of CLN are at their highest in the milk in the first few hours after birth. The complex was initially isolated from ovine colostrum by Janusz *et al.* in 1974 using electrophoresis methods to separate the components of the milk and the amino acid content was subsequently established using an automatic amino acid analyser (Janusz *et al.* 1974). The authors also found that CLN was highly proline rich with around 20% of the amino acids in the complex being proline but that it lacks both glycine and alanine and has a beta-sheet structure. Janusz *et al* (1974) also found that the constituent peptides had homology to the protein precursors annexin and β-casein and that CLN is susceptible to protease digestion but resistant to collagenase.

Since the first isolation of the ovine form of CLN a more efficient mode of purification has been developed using methanol (Kruzel *et al.* 2004) and bovine CLN has also been isolated. Moreover several of the constituent peptides of the polypeptide including the active colostral-val nonapeptide, (CVNP: Val-Glu-Ser-Tyr-Val-Pro-Leu-Phe-Pro) have been investigated for their individual effects *in vitro* and *in vivo* to try and isolate the more critical active components of CLN. A table

showing an amino acid analysis of CLN as percentages extracted from either ovine or bovine colostrum using two different methods (Janusz *et al.* 1974; Kruzel *et al.* 2001; Kruzel *et al.* 2004), is shown in Table 1 below (Boldogh and Kruzel 2008). The two step methanol extraction is more efficient than the original method and gives consistently pure CLN (Kruzel *et al.* 2004) whether it is derived from ovine or bovine sources (see Table 1). Despite the fact that the bovine form has a slightly different amino acid composition to the ovine form of CLN (Boldogh and Kruzel 2008) it has been found to have the same molecular weight as the ovine form, to contain around the same number and size of proteins and, moreover, when tested has shown the same biological effects as the ovine form (Bacsi *et al.* 2005).

Amino Acid	**Colostrinin (CLN)**		
	Ovine Original Method	**Ovine MeOH Method**	**Bovine MeOH Method**
Asp/Asn	3.42	2.80	5.13
Ser	5.66	5.05	6.94
Glu/Gln	15.48	15.77	17.99
Gly	3.15	3.03	2.95
His	2.54	2.14	3.28
Arg	2.32	3.34	1.62
Thr	5.73	5.30	4.32
Ala	2.78	2.13	3.06
Pro	21.07	22.50	20.79
Tyr	1.36	1.54	0.47
Val	9.27	11.10	8.15
Met	3.33	1.70	0.70
Lys	5.30	4.93	8.15
Ile	3.17	3.42	3.21
Leu	11.04	10.47	10.33
Phe	4.38	4.77	5.41

Table 1: To show the detailed amino acid composition of CLN with different modes of extraction. The numbers are percentages. From (Boldogh and Kruzel 2008).

The natural function of CLN *in vivo* and how it may become biologically active upon contact with the digestive tract is uncertain. However the effects of CLN on the immune system have been well documented (as detailed in Section 1.5.2 below) and it has been suggested that it may be related to the uptake of

immunoglobulins by the newborn or to 'kickstart' the immune system in the first few hours after birth. Other proline-rich polypeptides are known, in particular those isolated in hypothalamic neurosecretory granules, and these have been found to have effects on a range of processes including growth actor release and caspase 3 activity (Gladkevich *et al.* 2007) which implies that CLN could also have very diverse effects.

1.5.2. Colostrinin and effects on the immune system

As described in Section 1.5.1 CLN was initially of interest and investigated for its potential effects on the immune system. From these studies ovine CLN has been demonstrated to be able to stimulate the activation and growth of resting B lymphocytes, as well as affecting their differentiation, which can be assessed by plaque formation, with a similar same efficacy as LPS *in vitro* (Julius *et al.* 1988). CLN does appear though to stimulate a separate group of B lymphocytes to those induced by LPS as the effects are additive (Julius *et al.* 1988).

Moreover, ovine CLN alters the host-vs-graft response and has been seen to allow thymocytes that previously did not exhibit this response to be capable of producing the response in thymocyte cultures and *in vivo* in mice (Zimecki *et al.* 1982). Ovine CLN has also been shown to alter the binding properties of peanut agglutinin (PNA) to thymocytes, changing the properties of the cells from helper cells to suppressor cells and *vice versa* (Lisowski *et al.* 1988). This CLN-mediated effect on PNA binding has been found to involve β-galactosidase activity (Sokal *et al.* 1998) and although the mechanism of these actions is not more clearly known it has been suggested that the effects of CLN on thymocyte differentiation and

maturation may be mediated by CLN binding a receptor on the surface of thymocytes (Janusz and Lisowski 1993).

As well as regulating immune cell maturation CLN has been found to have other roles in regulating the immune system. In experiments utilising the injection of sheep red blood cells into mice in order to induce a humoral immune response it was found that ovine CLN given with the red blood cells was able to regulate the immune response differently depending on the current activation status of the immune system (Wieczorek *et al.* 1979). This regulatory effect means that if the immune response was high, CLN kept it under control by dampening it down and if the response was low then CLN caused a boost to 'kick start' the immune system. Furthermore, CLN was demonstrated to increase skin permability in guinea pigs and this appeared to be via effects on prostaglandins (Wieczorek *et al.* 1979).

Ovine CLN has further been demonstrated to be modest inducers of inflammatory cytokines, tumour necrosis factor (TNF)α and γ interferon (IFN) , in murine resident peritoneal cells, human peripheral blood leukocytes (Inglot *et al.* 1996; Kruzel *et al.* 2001) and in human whole blood cell cultures (Inglot *et al.* 1996) where CLN has been found to induce the release of interleukin-6 and interleukin-10 as well as TNFα and γIFN (Zablocka *et al.* 2001). CVNP has also been observed to mediate the cytokine inducing effects of CLN in leukocytes and whole blood cell cultures but with less efficiency than the whole CLN complex (Inglot 1996; Zablocka 2001) and the cytokine inducing activity of the nonapetide and hexapeptide of CLN appear to depend on whether they are monomers, dimmers or oligomers (Zablocka 2001).

Moreover, it has recently been reported by Boldogh *et al.* (2008), that CLN can help to decrease the severity of allergic responses such as mucin levels to

ragweed pollen and house dust mites without itself causing an allergic reaction in a mouse model of allergic inflammation (Boldogh *et al.* 2008).

CLN has therefore been shown *in vitro* and *in vivo* to have several effects on immune system cells and function (Janusz and Lisowski 1993; Zimecki 2008). The bovine form of CLN has also been found by a recent study using microarray analysis on TR146, human buccal mucosal cells to regulate the expression of many genes involved in pathways for immune system function including mediating the downregulation of c-jun-terminal kinase (JNK) which is activated in response to stress signals (Szaniszlo *et al.* 2009).

As described above the CVNP of CLN has been demonstrated to have most of the biological effects that the whole CLN compound has on the immune system (Staroscik *et al.* 1983) and in particular the sequence Pro-Leu-Phe has been shown to be critical for these immunological effects (Janusz and Lisowski 1993).

In addition to the above effects, bovine CLN has also been shown in microarray analysis of buccal mucosal cells to regulate genes involved in cell proliferation (Szaniszlo *et al.* 2009) and to inhibit the proliferation of PC12 cells via the activation of the cell-cycle regulator P53 and its downstream effecter, P21, (Bacsi *et al.* 2005). Moreover, both ovine and bovine forms of CLN bound to PC12 cells and stimulated neurite outgrowth which was associated with the increased expression of the neuronal development marker, GAP-43, and required activation of P53 and P21 (Bacsi *et al.* 2005). The similarity of the extent of neurite outgrowth induced by CLN and the molecules involved in its induction led the authors to speculate that CLN may act at a receptor and mediate a similar pathway to nerve growth factor (Bacsi *et al.* 2005). Ovine CLN has also been found to affect the vitamin D-induced differentiation of HL-60 moncocyte cell line but only when given before or with

vitamin D and therefore appears to affect an early stage in the process (Kubis *et al.* 2005).

1.5.3. Colostrinin and oxidative stress

Research has accumulated to show that CLN can protect against oxidative stress related cellular damage *in vitro* (Boldogh and Kruzel 2008). Bacsi *et al.* (2006) found that CLN protects Chinese hamster V79 fibroblast cells from oxidative stress-induced mutations as well as spontaneous mutation and UV or chemically-induced mutation. Furthermore, the same authors demonstrated that CLN significantly increases the lifespan of both senescence sensitive and senescence resistant murine diploid cell cultures by around 30% via reducing ROS accumulation in the mitochondria and therefore preventing mitochondrial damage in these cells (Bacsi *et al.* 2007).

Ovine CLN has also been shown in the PC12 cell line to prevent 4-HNE protein adduct formation, prevent 4-HNE –induced increases in JNK and P53 as well as decreasing 4-HNE-induced glutathione depletion and ROS production as seen using 2'7'-dichlorofluorescein (DCF) (Boldogh *et al.* 2003). Both the whole ovine CLN complex and CVNP have been found by Zablocka *et al.* (2000), to inhibit lipopolysaccaride (LPS)-induced nitric oxide (NO) release in whole blood cells (Zablocka *et al.* 2005). However, Mikulska and Lisowski (2003) found that in the human monocyte/macrophage cell line, THP-1, only the nonapeptide, but not whole ovine CLN could prevent LPS induced NO release. Furthermore CLN has been found to prevent increases in ROS levels in response to H_2O_2 or Aβ in SH-SY5Y cells (Boldogh and Kruzel 2008).

It is now thought that these antioxidant effects that are mediated by CLN appear not to be due to a direct antioxidant action of CLN in removing ROS but to be

initiated via the inhibition of molecules which will lead to the production of ROS (Stewart 2008).

1.5.4. Colostrinin and Alzheimer's disease

The research into the ability of CLN to affect the release of cytokines discussed in Section 1.5.2 involved the participation of AD patients that were given CLN orally (Inglot *et al.* 1996). CLN was later reported by a subset of the patients participating to also have a psycho-stimulatory effect (Inglot *et al.* 1996) and this led to interest into whether CLN may be beneficial for patients with dementia. Therefore two groups have conducted separate, placebo-controlled clinical studies which were set up to investigate whether CLN had a beneficial effect in AD patients at various stages of Alzheimer's disease (Leszek *et al.* 1999; Leszek *et al.* 2002). Lezek *et al* (1999) carried out a trial with 46 AD patients separated into three receiving 100μg ovine CLN, selenium or placebo tablets orally every other day for two weeks followed by three weeks off over 10 cycles. The authors found that 8 out of 15 patients on CLN showed improvement in the MMSE test and the other 7 stabilised (Leszek *et al.* 1999). In comparison no improvement was observed in patients on selenium or placebo tablets although 13 of 15 patients on selenium and half of those on placebo stabilised (Leszek *et al.* 1999). These authors later carried out a longer trail in which 33 patients were given ovine CLN in the same treatment regimen as detailed for the first study for between 16 and 28 months and again found that CLN led to an improvement in MMSE test scores (Leszek *et al.* 2002). CLN was found to be particularly beneficial to patients in the early stages of the disease with relatively mild symptoms and slightly less beneficial to those at more advanced stages of disease. Furthermore the authors found that there were only mild and very short lived side effects such as anxiety and insomnia (Leszek *et al.* 1999; Leszek *et al.* 2002).

An independent study was then conducted by Bilikiewicz and Gaus (2004) to verify the results obtained by Leszek *et al* (1999). The authors had 105 AD patients and treated half of them with 100μg ovine CLN and half with placebo tablets every other day for two weeks followed by three weeks off over 15 weeks before repeating this 15 week treatment cycle giving all of the patients CLN (Bilikiewicz and Gaus 2004). Assessments were carried out on the patients at 15 weeks and 30 weeks using ADAS-cog, Clinical Global Impression of Change, MMSE, ADAS-non cog and Instrumental Activities of Daily Living and showed a stabilising effect with CLN according to ADAS-cog and Instrumental Activities of Daily Living (Bilikiewicz and Gaus 2004). As described in Section 1.1.10 more, larger clinical trials than these detailed for CLN have been carried out to investigate the efficacy of acetylcholine esterase inhibitors in AD and have shown efficacy even for more severe AD, where CLN has demonstrated far more of an effect for patients with mild AD (Birks 2006; Birks and Harvey 2006; Loy and Schneider 2006). Acetylcholine esterase inhibitors have been demonstrated to commonly lead to side effects which have resulted in people leaving studies (Birks 2006). CLN, which despite occasional more serious side effects such as psychosis and gastrointestinal disturbance appears to be very well tolerated with only mild, short lived side effects (Rattray 2005).

Further to the beneficial effects of CLN treatment seen in AD patients CLN has been shown to improve memory in rats. CLN has been demonstrated to improve spatial memory in aged rats as seen by the Morris water maze task (Popik *et al.* 1999; Popik 2001; Popik *et al.* 2001). CVNP has also been shown to affect spatial memory (Popik *et al.* 2001). However, the effect on spatial memory differs between CVNP and the whole peptide. CVNP appears to delay the extinction of memories (Popik *et al.* 2001) rather than improve the acquisition of these spatial memories as the whole polypeptide complex has been shown to be able to do. There is also evidence from

the passive avoidance learning test in one-day old chicks showing that CLN can enhance long term memory (Stewart and Banks 2006) but only the whole polypeptide complex and not individual constituent peptides of CLN could mediate these effects on chick memory (Stewart and Banks 2006).

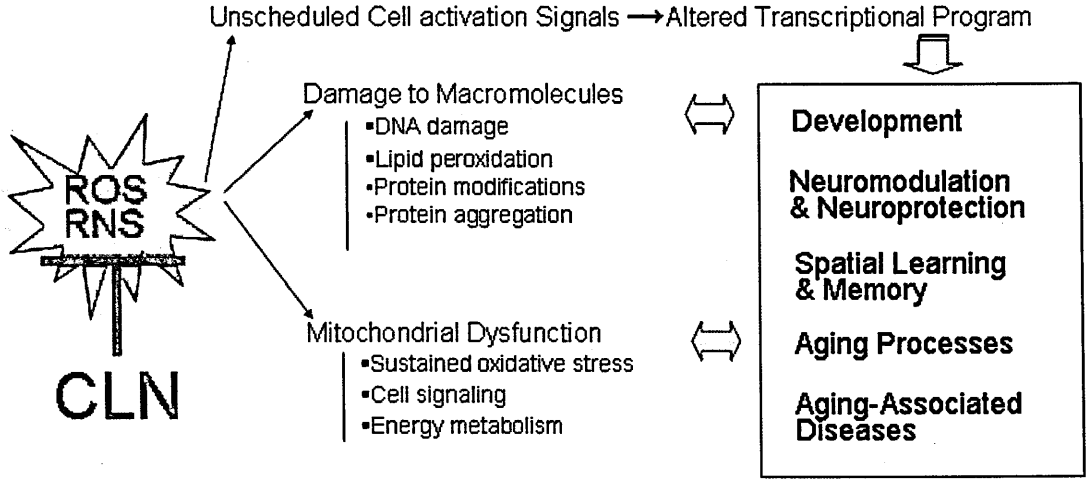


Figure 1-9: Modes of action of CLN that may prevent pathology. From Boldogh and Kruzel (2008).

It is not known exactly how CLN exerts these positive effects in the CNS on memory and in AD patients, or indeed how CLN may be processed when it's given orally. Some potential modes of action are illustrated in Figure 1-9. It may be that a constituent peptide can cross into the CNS or possibly the effects are related to cytokine release or cell signalling effects. Much work has been done to study its effects and potential mechanisms including an antioxidant effect (Stewart 2008). Oxidative stress is known to be very important in the development of AD and disease progression as discussed in Section 1.3.2 and CLN has been demonstrated in several

studies to exert antioxidant effects (as detailed in Section 1.5.3). Also as previously mentioned CLN has been observed to be a modest cytokine inducer, particularly of TNFα and INFγ (Inglot *et al.* 1996; Blach-Olszewska and Janusz 1997; Zablocka *et al.* 2001). It is possible that CLN may be able to regulate release of anti-inflammatory cytokines as well as the pro-inflammatory TNFα and INFγ although this has not as yet been investigated. This ability to modulate the release of cytokines may therefore be another possible mechanism of action of CLN.

One potential mode of action for CLN that is directly linked to the pathology seen in AD patients is the discovery that CLN can hinder the aggregation of beta-amyloid (Schuster *et al.* 2005). This ability of CLN to affect aggregation could be central to CLN's ability to benefit AD patients as the aggregation state of Aβ has been demonstrated *in vitro* and *in vivo* to be crucial to the level of neurotoxicity that Aβ causes. Furthermore Szaniszlo *et al.* (2009) demonstrated that bovine CLN can upregulate the Aβ cleavage molecule bleomycin hydrolase and can downregulate the expression of the Aβ precursor APP. Together these data suggest that CLN may prevent the accumulation of Aβ plaques. Moreover, as discussed in Section 1.1.1., the phosphorylation and subsequent aggregation of Tau may exacerbate toxicity in AD and bovine CLN has been shown by microarray analysis on TR146, buccal mucosal cells to downregulate the gene expression of protein kinase A (PKA) which is involved in the phosphorylation of Tau (Szaniszlo *et al.* 2009).

This introduction has described the known effects of CLN and the potential benefits of the complex to patients with AD but it is also clear that there is a lot more to learn about the mechanism of action of CLN. The aim of this research was therefore to further investigate the mechanism of action of CLN using *in vitro* models.

The toxicity of Aβ and the vulnerability of the hippocampus as well as the relevance of oxidative stress in AD and other neurodegenerative diseases, has been discussed. Therefore the ability of bovine CLN to eleviate Aβ-induced or ROS-induced toxicity in hippocampal cells *in vitro* was investigated. Furthermore, the effects of bovine CLN on ROS production, antioxidant defence and apoptosis in primary hippocampal cells and the B50 neuronal cell line were examined.

Chapter 2

Materials and methods

2.1. Materials and antibodies

Full details of the materials and consumables used are in appendix 1, Section 8.1 and the primary and secondary antibodies are in appendix 1, sections 8.2.1 and 8.2.2 respectively.

2.2. Animals

Rats of the Sprague Dawley strain were used at Postnatal day 4 (P4) and E18. The litters usually contained about 12 pups. The mothers were kept in a 12 hour light/dark cycle, fed *ad libitum* with standard rat pellets and had free access to water. Animals were sacrificed by anaesthetic overdoes as approved as a Home Office approved Schedule 1 procedure.

2.3. Dissociated hippocampal culture

Primary hippocampal slice cultures (Bruce *et al.* 1996; Lezoualc'h *et al.* 1996) and dissociated cell cultures (Harris *et al.* 1995; Maiese *et al.* 1995; Kittur *et al.* 2002) from embryonic and early postnatal rat pups are extensively used as a model culture system for cell death and protection experiments.

A protocol for the culture of dissociated rat hippocampal cells was established, based on a method from a personal communication from Frances Colyer of The Life Sciences department, Open University. This method has been used previously by others in our laboratory and has been considered to be a reliable method for the culture of hippocampal cells. It is similar to the method used by

Banker and Cowan (1977). Prenatal (E18) or early postnatal (P4) rats were used for the hippocampal cultures because it has previously been found that cells from young animals produce cultures with improved viability. This last point is discussed in more detail in Chapter 3, Section 3.2.1.

2.3.1. Removal of the hippocampus

For cultures from P4 animals, 8-12 rat pups were used for each preparation. After decapitation the brain was accessed by an incision along the skin and skull and was removed using a spatula and then placed into dissection media (Dulbeccos Modified Medium (DMEM) medium with 1% penicillin/streptomycin).

For cultures from E18 animals, tissue from an entire litter was used for each preparation. The mother was given an overdose of anaesthetic and an incision was made into the abdominal cavity and through the uterus wall to remove the pups. The brain was then removed from the pups by cutting through the skull beneath the brain. The hippocampi were located by separation of the cerebral hemispheres and the overlying cortex was pulled away with forceps. The hippocampi were then very gently excised using fine curved forceps, placed into fresh media (Banker and Cowan 1977; Stoppini *et al.* 1991) and for P4 cultures the hippocampi were chopped into 3 or 4 pieces.

2.3.2. Dispersion of cells

After removing the hippocampi the DMEM medium was removed and replaced with 1ml E1 solution (Earl's balanced salt solution (EBSS) with 6mg/ml papain, 1mg/ml cysteine, EDTA solution (1.64mM NaOH, 2mM EDTA) and 1mM β-mercaptoethanol). The hippocampi were incubated in E1 at 37°C in a shaking

water bath for 25 minutes. E1 solution was then removed and replaced with 1ml of E2 solution (DMEM with 1mg/ml chicken egg white trypsin inhibitor and 1mg/ml DNAase). The tissue was dispersed by trituration with a glass Pasteur pipette that had been flamed at the tip. The E2 solution was then made up to 10ml and the suspension spun at 800rpm for 5 minutes.

2.3.3. Cell counting and plating

Once the solution had been spun, the supernatant was removed and the pellet resuspended in 2ml medium containing horse serum (Basal Medium Eagles (BME), 0.5% glucose, 1mM sodium pyruvate, 1% penicillin streptomycin, N2 supplement and 10% horse serum (HS)). The solution was then made up to 10ml with more media and the cells were counted in a haemocytometer using trypan blue to stain any dead cells. The cells were then plated at an appropriate density for the experiment (20,000 – 80,000 cells/well) on poly-lysine coated 19mm coverslips in 12 well plates.

2.3.4. Maintenance of cultures

The media were changed 24 hours after the cultures were set up. On the third day *in vitro* (3 *DIV*) the media were replaced with media including the supplement B27, which contains antioxidants, vitamins, essential fatty acids and hormones (Brewer, 1997), but without horse serum.

2.4. B50 cell line

The B50 cell line is a rat neuronal cell line produced from a chemically-induced neuroblastoma. The cell line has been well used in cellular toxicity and death experiments including investigation into the effects of hydrogen peroxide on cells in culture (Lai *et al.* 1993; Honma *et al.* 1997; Iwata *et al.* 1998). The line was purchased frozen from the European Collection of Cell Cultures (ECACC). The cells were maintained in DMEM with 2mM glutamine and 10% fetal calf serum (FCS) at 37°C/ 5% CO_2 and passaged at a 1:3 dilution twice weekly using trypsin-EDTA to release the cells. Cells were frozen in DMEM with 20% FCS and 10% DMSO in 1ml aliquots and kept at -80°C for 48 hours before being stored in liquid nitrogen.

2.5. Preparation of bovine CLN

Bovine CLN received in batches from Regen Therapeutics was dissolved in Phosphate Buffered Saline (PBS) at 2mg/ml and then diluted as appropriate for treatment. The natural concentration of CLN is unknown and therefore concentrations were based those used in previous experiments.

2.6. Preparation of menadione

Menadione from Sigma Aldrich (M5625) was dissolved in ethanol at 100mM and then diluted in the culture media used on the cells in that specific experiment to

between 1μM and 20μM for treatment of the cultures. This equates to between 0.001% and 0.02% ethanol in the culture.

2.7. Preparation of beta-amyloid

$A\beta_{1-42}$ was dissolved in saline and then diluted to 1mg/ml in phosphate buffered saline (PBS) and incubated for 48hours at 37°C/5%CO_2 with or without bovine CLN. This pre-incubated stock was then diluted in culture media to the required concentration prior to treatment.

2.8. Immunohistochemistry

2.8.1. Cell preparation

The primary hippocampal cultures on glass coverslips were fixed in 4% paraformaldehyde/PBS pH 7.4 for 1 hour at room temperature (RT) and washed twice with PBS (Adamec *et al.* 2001; Alvarez *et al.* 2004).

2.8.2. Optimisation of immunolabelling

Several concentrations of the antibodies used were tested on the primary hippocampal cultures in order to optimise staining. Staining was considered optimised when there was clear staining of the cells being labelled without background fluorescence. Concentrations between 1:100 and 1:1000 were tested at RT overnight or for 48hours for the anti-MAP-2 antibody; 1:1000 for 48hours was

found to be optimum. 1:100, 1:1000 and 1:2000 at RT were tested for the anti-GFAP antibody and 1:1000 overnight was found to be optimum. Both anti-2, 3-cyclic nucleotide 3-phosphodiesterase (CNPase) and anti-CD11b were tested at 1:200, 1:250 and 1:400 at RT; the dilution of 1:250 overnight was chosen as optimum. Controls using only the secondary antibody without the primary antibody were also carried out for all antibodies and showed no background reactivity. Further controls that could have also been carried out are reagent only controls to check for background peroxidise activity and testing antibody specifity by blocking with a closely related antigen to the antigen that the antibody is against.

2.8.3. GFAP immunolabelling

GFAP is an astrocytic intermediate filament protein that has been well-characterised as a marker for astrocytes in the CNS (Eng *et al.* 2000) including those in embryonic (Assis-Nascimento *et al.* 2007) and adult rat hippocampal cultures (Brewer 1997). The antibody chosen (Dako-Cytomation 20334) for these experiments has been demonstrated to label rat and mouse astrocytes after fixation with paraformaldehyde (Castellano *et al.* 1991).

Non-specific binding was blocked with 10% donkey serum diluted in antibody diluting solution (ABDS) which contained PBS with 1mg/ml lysine, 0.01% BSA, 0.05% azide to preserve cultures and 0.1% triton X100 for permeabilisation. After removal of the blocking solution anti-GFAP antibody (1:1000 in ABDS) was applied overnight at RT with shaking. The biotin-strepavidin fluorescein secondary system was then used to visualise the immunolabelling. Following three 10 minute washes in PBS, anti-rabbit biotinylated antibody (1:250 in ABDS) was applied for 1 hour at RT with shaking followed by a further three washes in PBS and incubation with strepavidin fluorescein (1:100 in ABDS) for 1 hour at RT with shaking. A foil

cover was also used at this last stage to protect the fluorescence from light-induced fading.

2.8.4. MAP-2 immunolabelling

MAP is one of the most abundant microtubule associated proteins that is expressed in neurons of the both the CNS and PNS and it is expressed throughout development (Teng *et al.* 2001). MAP-1 and MAP-2 are both localised to nerve cells but MAP-2 has been more extensively studied and has, in particular, been used as a neuronal marker in hippocampal cultures (Adamec *et al.* 2001; Yu *et al.* 2007). The MAP-2 monoclonal antibody was therefore chosen as the neuronal marker because it is a pan neuronal marker that has been demonstrated to effectively label neurons in both embryonic and neonatal rat neuronal cultures and it is compatible with paraformaldehyde fixation (Adamec *et al.* 2001; Yu *et al.* 2007).

Non-specific binding was blocked using 10% goat serum and then anti-MAP-2 (1:1000) antibody was applied for 48 hours at RT with shaking followed by three 10 minute washes in PBS and goat-anti-mouse amino-methyl-coumarin-acetate (AMCA) conjugated secondary antibody (1:100) for 1 hour with shaking and a foil cover.

2.8.5. CNPase and CD11b immunolabelling

CNPase is an oligodendrocyte marker and CD11b is commonly used as a microglial marker.

Non-specific binding was blocked using 10% goat serum and then anti-CNPase (1:250) antibody or anti-CD11b antibody (1:250) was applied for 48 hours at RT with shaking followed by three 10 minute washes in PBS and goat-anti-mouse

rhodamine conjugated secondary antibody (1:100) for 1 hour with shaking and a foil cover.

2.8.6. Cell counting

Double labelling with GFAP (1:1000) and MAP-2 (1:1000) or GFAP (1:1000) and CNPase (1:250)/CD11b (1:250) antisera was carried out and coverslips were mounted onto microscope slides with Citifluor (glycerol/PBS) mountant. The immunofluorescence was then visualised using an Olympus BX61 fluorescence digital photomicroscope. The FITC filter (excitation 494, emission 518) was used to visualise the fluorescein (excitation 494, emission 521) conjugated GFAP labelling while AMCA (excitation 349, emission 448) conjugated, MAP-2 labelling was observed under the DAPI filter (excitation 345, emission 445) and rhodamine (excitation 550, emission 570) conjugated CNPase and CD11b labels were visualised using the texas red filter (excitation 595, emission 615).

After some preliminary tests using several counting methods with fewer or more frames and in different forms, circular grids or cross shaped grids, a square grid of 100 frames was chosen as the optimum. Positive cells were counted manually for each cell type individually using the grid system on the stage manager function on the microscope at x400 magnification. One hundred grid squares (347.64μm by 260.73μm) covering $9mm^2$ of the coverslip in total were counted for each coverslip and there were at least three replicates for each condition per experiment. Only healthy/viable looking cells; smooth, round neurons and flat, filamentous astrocytes with a clearly visable nucleus, that were clearly positively stained were counted and the cells on the lines at the left hand side and bottom of the square were not counted in order to avoid recounting (see Figure 2-1). Digital images were taken at x200 or x400 magnification using AnalySiS software.

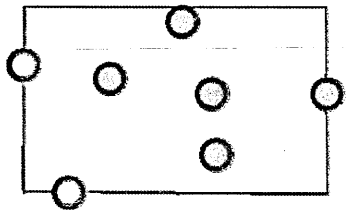


Figure 2-1: Illustration of the counting procedure followed using one frame out of the 100 frames used for each coverslip as an example. The cells in red on the left and bottom lines of the frame would not have been counted. Any cells in the positions of the blue cells would have been counted if they were positively stained and met morphological criteria.

2.9. Electron microscopy (EM)

Dissociated hippocampal cultures from P4 rats were prepared as described in 2.3 and treated at *DIV* 6 with media alone or with 100ng/ml CLN for 24 hours. Cultures were then fixed in 2% paraformaldehyde/2% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.4 for 1hr and post fixed in 1% osmium tetroxide in 0.1M sodium cacodylate for 1hr before washing in sodium cacodylate buffer.

Cultures were then dehydrated in graded ethanol with 10 minutes in 50, 70, 80, 95% ethanol and then 2 x 10 minutes in 100% ethanol before being put in 100% ethanol containing molecular sieve beads for 10 minutes to absorb moisture. The coverslips were then placed in 50:50 100% ethanol:resin (Epon 812/Araldite M epoxy resins) for 20 min.

Each coverslip was removed from the culture plate using forceps and the resin/ethanol was drain off onto filter paper. The ethanol was then allowed to evaporate and each coverslip was placed culture side down onto the surface of a mould filled with resin. The resin was polymerised by heating for 48hrs in an oven at 60°C and the mould was removed. The glass coverslip was then removed by 2 hrs immersion in hydrofluoric acid before being rinsed with water and allowed to dry.

Preparations were then viewed under a light microscope through the resin block to locate cells and the resin blocks were then trimmed with a glass knife and 1μm thick sections cut, collected on a glass slide and stained with 1% toluidine blue before viewing in a light microscope to definitively confirm the presence of cells. For electron microscopy serial sections of grey/white colour (60–70 nm) were cut with a Diatome diamond knife and collected using Pioloform-coated slot copper grids. Sections were counterstained with saturated ethanolic uranyl acetate, followed by lead citrate, and were then placed in a rotating grid holder to allow uniform orientation of Sections on adjacent grids in the JEOL 1010 electron microscope. sections were viewed at a magnification of 6000 on the microscope screen and captured digitally with a GATAN Bioscan camera. Images were printed at a final magnification of x20K.

2.10. MTS cytotoxicity assay

The MTS cytotoxicity assay uses the reduction of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), to soluble formazan as a measure of the mitochondrial activity within cell cultures. To carry out this assay B50 cells were plated at 5,000 cells/well in 96 well culture dishes, treated with CLN immediately after plating or on *DIV*1. Menadione treatment was carried out on *DIV*1. On *DIV*2 the MTS solution was added to the culture medium in a 1:5 dilution for 1 hour at 37°C before the assay being read at 490nm in a plate reader.

2.11. Dichlorofluorescein diacetate and reactive oxygen species

2.11.1. Dichlorofluorescein diacetate (DCFH-DA)

DCFH-DA is a compound that can freely enter cells where it is converted to insoluble DCFH by esterases. DCFH can then react with peroxides to produce the fluorescent compound DCF which can be viewed under the microscope. The fluorescence can be quantified as a measure of relative ROS production (Harris *et al.* 1995; Xie *et al.* 1996; Sultana *et al.* 2005).

2.11.2. Cell preparation and treatment

B50 cells were plated at 200K cells/well on coverslips in 6 well culture plates and the experiment was begun on *DIV*2. The cells were treated with 10μM menadione with or without 1μg/ml CLN for 24 hours before addition of 25μM DCFH-DA for 1 hour.

2.11.3. Confocal microscopy and analysis

Images were taken of 3 separate fields in the centre of each coverslip on the Leica DMIRBE confocal microscope. The FITC channel was used to excite the fluorescence (excitation 494, emission 518) and the x60 oil immersion objective was used to view the samples and take images. A scanning distance of 10μm was chosen in order to pass through the entire monolayer and twenty scans were taken over this distance and then combined to obtain high quality images. Quantification was then carried using mean and maximum intensity values of the images which were obtained for each field of view using Volocity software.

2.12. Western blot

2.12.1. Cell preparation

In order to ensure sufficient protein yield for the Western blot, cells were plated in large 6 well culture plates at high densities of 400,000 cells/well for primary hippocampal cells and 100,000 cells/well for B50 cells and treated as described for the individual experiment. After treatment cells were lysed with radio-immunoprecipitation assay (RIPA) lysis buffer containing protease inhibitors to prevent destruction of the protein, sonicated and then spun to remove debris. Protein content of the samples was then assayed using a detergent compatable (DC) colorimetric protein assay protein assay. Primary hippocampal cell lysate gave low protein yields and 20μg of protein onto the gels was considered sufficient. However, much higher yield was obtained from the B50 cell lyaste and therefore 50μg of protein was used as more protein will allow clearer bands.

2.12.2. SDS PAGE

Cell lysate was diluted in ddH_2O to the appropriate protein concentration, 4x Laemmli sample buffer (SDS, Tris, glycerol, β-mercaptoethanol and bromophenol blue) was added to the samples and samples were boiled for 10 minutes. After being spun down the samples were loaded onto a 15% SDS polyacryamide gel. The gel was run in Tris/glycine/SDS running buffer at 100V for 2 hours at RT and transferred in transfer buffer containing Tris/glycine and 20% ethanol onto nitrocellulose membrane overnight at 4°C.

2.12.3. Western blotting

Western blots for SOD1 or Cdk5 were then performed. After blocking non-specific binding on the nitrocellulose membrane by 1 hour incubation in 5% dried skimmed milk (Marvel) in PBS/Tween at RT anti-SOD1 (Abcam ab51254) or anti Cdk5 (Santa Cruz SC-173) antibodies were then applied to the membrane at 1:25,000 in 50ml 5% milk/PBS/Tween, or 1:200 in 2ml 5% milk/PBS/Tween, respectively for 1 hour at RT with shaking. The membrane was then washed in 5% milk in PBS/Tween for 1 hour with shaking and several changes of the solution before being incubated in anti-rabbit horseradish peroxidise conjugated secondary for 1 hour at RT with shaking. A further two washes in 5% milk in PBS/Tween, one wash in PBS/Tween and finally a PBS only wash were then carried out following the secondary antibody incubation.

ECL chemiluminescent reagent was used to detect horseradish peroxidase on the secondary antibody. The ECL reagents A and B were mixed in a 1:1 dilution and the membrane was shaken in the solution for 2 minutes then placed in clear plastic in a film case. Developing film was then exposed to the blot for an appropriate time and an EXOMAT developing machine in a darkroom was used to develop the film.

A second blocking step in 5% marvel/PBS Tween was then carried out and rabbit anti-actin antibody applied overnight at 4°C with shaking before washing in 5% milk in PBS/Tween as above and incubating in anti-rabbit secondary antibody for 1hr at RT with shaking. The final washing procedure and ECL developing was then carried out as above.

2.12.4. Quantification

The developed Western blot films were scanned as greyscale images and the software program ImageJ was used to carry out densitometry analysis on these

images. This analysis was carried out by selecting the same area over each separate band and using the analyse gels function on the ImageJ software to obtain a plot. The area under this curve gave desnity values for each band. The density value for the observed protein band was then divded by the value for the corresponding actin band to give an actin normalised band density.

2.13. Fluorescence Activated Cell Sorting analysis

FACS allows the separation and counting of cells in a given sample according to size and fluorescence.

Primary hippocampal cells from E18 rats and B50 cells were prepared for FACS analysis. Cells were plated in 6 well plates at 400,000 cells/well for primary cells and 100,000 cells/well for B50 cells. The cultures were treated as described for the specific experiment and then released with trypsin. This cell suspension was spun at 4,000rpm for 5mins, washed in PBS and then resuspended in 0.4% paraformaldehyde for 30mins.

For the intracellular protein active caspase 3 the procedure was carried out as follows. After fixation the cells were spun and then washed twice with spinning (4,000rpm for 5mins) in 0.01% triton X/PBS solution to permeabilise the cells before incubation in an anti-activated caspase 3 antibody which binds to both subunits of the enzyme (Abcam, ab13847. 1:500 in 0.01% triton-X/PBS) for 60mins. The cells were then washed in 0.01% triton X/PBS solution twice again and the secondary donkey-anti rabbit biotinylated antibody was applied for 40mins (1:250 in 0.01% Triton-X/PBS). Following two more washes the streptavidin fluorescein antibody was added

for 40mins at RT (1:100 in 0.01% Triton-X/PBS). The cells then had two final washes and were resuspended in PBS for the analysis on the FACS analyser.

2.14. Analysis and statistics

Quantitative data were obtained for all experiments in the form of cell counts, absorbance values for MTS, intensity values for DCF, semi-quantitative densitometry values expressed as percentage of control for Western blots and percentages of stained cells in 10,000 for FACS analysis. Densitometry values were expressed as percentage of control because this was considered the clearest way to illustrate this data due to variations in the control band density between experiments. Where relevant the mean of the replicates for each condition within experiments was then calculated. The overall mean and standard errors were then calculated by pooling the results of repeat experiments and histograms were produced.

Advice regarding appropriate statistical tests was obtained from Dr. Faria in the statistics department at The Open University. Normality of the data was established using plots of the data and variance within groups was tested using homogeneity of variance in the SPSS statistics package version 16 to check if the data met the criteria for parametric testing. For data that was considered to meet the criteria for parametric tests a one-way ANOVA was used in order to analyse the variance in the means between groups with a Bonferroni *post-hoc* test as this is a simple, commonly used *post-hoc* test which is flexible and therefore suitable for a range of data sets. Where the data did not meet the criteria for a parametric test the non-parametric Kruskal-Wallis test for difference between more than two groups was carried out and if this showed a significant difference the Mann-Whitney, non-

parametric test for comparisons between two groups was used as a *post-hoc* test.

These tests were carried out using the SPSS statistics package version 16.

Chapter 3

Characterisation of dissociated hippocampal cultures and trophic effects of CLN

3.1. Aims

The aim of these experiments was two-fold;

a) to establish the cell types present, with relative proportions, in the primary hippocampal cultures that were to be used as a model culture system and

b) to investigate whether bovine CLN alone has any adverse or trophic effect on these cells in culture.

In order to achieve these aims primary dissociated hippocampal cultures were set up from both P4 and E18 Sprague Dawley rats and cultures were treated with bovine CLN at a range of concentrations for 24 hours.

Immunostaining was carried out using antibodies against the neuronal marker MAP-2 and the astrocytic marker GFAP as well as microglial (CD11b) and oligodendrocyte (CNPase) markers.

Analysis by EM was also carried out on dissociated cultures from P4 rat hippocampus.

3.2. Introduction

3.2.1. Dissociated rat hippocampal culture as a model system

Dissociated hippocampal cultures were chosen as a model system for the investigation of the effects of CLN on cells in culture and analysis of the mechanism of action of CLN in its beneficial effects for AD patients. This culture system was chosen for several reasons.

Firstly, the limbic system and in particular the hippocampus have been shown to be areas that are among the first and most severely affected areas in the AD brain. Furthermore, as discussed in Chapter 1, Section 1.5.4, CLN has been demonstrated to improve the hippocampal dependent, spatial memory in aged rats (Popik *et al.* 1999) and associative learning in day old chicks (Stewart and Banks 2006) which is mediated by the chick brain area that is equivalent to the hippocampus. The hippocampus is therefore an area of great importance in protection against AD progression and in particular for the protective effects of CLN.

Secondly, dissociated rat hippocampal cultures have been extensively used as a model system for a wide variety of studies including the investigation of synaptic formation (Yamamoto *et al.* 2005) the toxicity of viruses (Brunner *et al.* 2007) and neurotrophic effects (Heaton *et al.* 1994; Okuda *et al.* 1994). In particular these cultures have been a major model used *in vitro* to analyse Aβ induced toxicity (Whitson and Appel 1995; Puttfarcken *et al.* 1996; Paula-Lima *et al.* 2005). These cultures have also been used to study oxidative stress mediated toxicity (Zhang *et al.* 2001) although organotypic hippocampal cultures have more frequently been used for these studies (Lezoualc'h *et al.* 1996; Masino *et al.* 1999; Avshalumov and Rice 2002; Niiyama *et al.* 2005).

These dissociated cells have also been demonstrated to maintain properties of *in vivo* hippocampal cells when *in vitro*. Banker and Cowen (1979) showed that

approximately 45% of hippocampal cells in culture closely resembled normal pyramidal cells and synapses in culture have been shown to be similar to those at a corresponding developmental time *in vivo* (Boyer *et al.* 1998). Increases observed in Ca^{2+} channels of cells in culture have been found to correspond to patterns seen in neurons *in vivo* (Porter *et al.* 1997). Furthermore, the developmental pattern of $GABA_A$ receptor expression has been found to be similar *in vitro* and *in vivo* (Brooks-Kayal *et al.* 1998).

Embryonic and postnatal rat hippocampi were chosen as the source of cells rather than those from adult rats because the cells from younger animals have been more commonly used and have been found to have improved survival rates in culture. Cells isolated from older animals have proven much more difficult to culture although there are methods that have been found to work for the culture of hippocampal cells from aged animals (Brewer 1997; Evans *et al.* 1998). In hippocampal cultures it has been demonstrated that these cells from older animals have similar properties to cells from embryonic rats although the embryonic cells fired more action potentials in response to a depolarising current (Evans *et al.* 1998).

3.2.2. The effects of CLN on cell survival in culture

As discussed in Chapter 1, Section 1.5.2, CLN has been found to have some regulatory effects on the immune system, affecting the activation and differentiation of cells in the immune system as well as stimulating neurite outgrowth in PC12 cells (Bacsi *et al.* 2005) and regulating genes involved in cell proliferation (Szaniszlo *et al.* 2009). CLN may therefore be hypothesised to mediate a trophic effect on primary hippocampal cells in culture leading to neurite outgrowth and/or increased cell proliferation. These experiments were designed to investigate that hypothesis.

This work focussed on both neurons and astrocytes. The main interest here was neuronal survival with reference to neurodegeneration in AD and astrocytes have been demonstrated to directly affect neuronal survival via trophic support and removal of toxins (Gasser and Hatten 1990; Ye and Sontheimer 1998 and reviewed in Markiewicz and Lukomska 2006). Furthermore a role for astrocytes has been established in the development of amyloid plaques and AD (Nagele *et al.* 2004; Farfara *et al.* 2008), as well as in affecting the severity of Aβ-induced toxicity *in vitro* (Domenici *et al.* 2002; Abramov and Duchen 2005; Ramirez *et al.* 2005).

3.3. Methods

3.3.1. Characterisation of cultures and the effect of CLN on cell numbers

Dissociated hippocampal cultures from E18 and P4 rats were set up using the methods described in Chapter 2, Section 2.3. Cells were plated at 40,000 cells/well in 12-well plates.

Bovine CLN was prepared as described in Chapter 2, Section 2.5 and diluted in culture media to concentrations of CLN between 1ng/ml and 100ng/ml. Control cultures were treated with culture media alone. Cultures were treated on *DIV*3 with at least 3 replicate coverslips, per condition, per culture.

Twenty four hours after treatment, immunolabelling, cell counting and analysis were carried out as described in Chapter 2, Section 2.8.

3.3.2. Electron microscopy

Dissociated hippocampal cultures were set up as described in Chapter 2 Section 2.3 and plated at 80,000 cells/well.

In order to allow a dense monolayer of cells to form with and allow for the maturation of synapses the cells were maintained in culture until *DIV*6. The cultures were then treated with culture medium alone or 100ng/ml bovine CLN diluted in culture medium. Twenty four hours after treatment, cultures were fixed and processed for EM as described in Chapter 2 Section 2.9.

3.4. Results

3.4.1. Characterisation of primary hippocampal cultures

Immunolabelling was optimised on cultures at *DIV*4 as detailed in Chapter 2, Section 2.8.2 and showed that cultures from E18 hippocampus contained mainly MAP-2 positive neurons with small numbers of GFAP positive astrocytes (see Figure 3-1A and C) but no CD11b positive microglia were present. In cultures from E18 hippocampi there appeared to be some positive CNPase staining for oligodendrocytes, however, this was faint and from inspection of cell morphology and phase contrast images it was decided that this staining was not specific (see Figures 3-1E).

Cultures from P4 hippocampi consisted largely of MAP-2 positive neurons and GFAP positive astrocytes (see Figure 3-2 A and C). There appeared to also be small numbers of microglia. In cultures from P4 hippocampi there appeared to be some positive CNPase staining, however, this was again feint and it was decided that this staining was not specific (see Figures 3-2E).

In cultures from both P4 and E18 rat hippocampus there were large numbers of MAP-2 positive neurons present along with strongly GFAP positive astrocytes (see Figures 3-3 and 3-4) and by *DIV*4 the cultures were well established, neurons were rounded and had extended long, sometimes multiple, processes (see Figures 3-3A and B). GFAP positive astrocytes were present in both flattened and stellate forms (as described in Gasser and Hatten, 1990. see Figures 3-4C). The proportion of GFAP positive astrocytes and MAP-2 positive neurons differed between cultures originating from E18 hippocampus and those from P4 hippocampus (see Figures 3-3 and 3-4). In cultures from P4 rat hippocampus the astrocytes appeared to cover the neurons and some cells were also observed which were of 'astrocytic' type morphology but which were not strongly GFAP positive (see Figure 3-4).

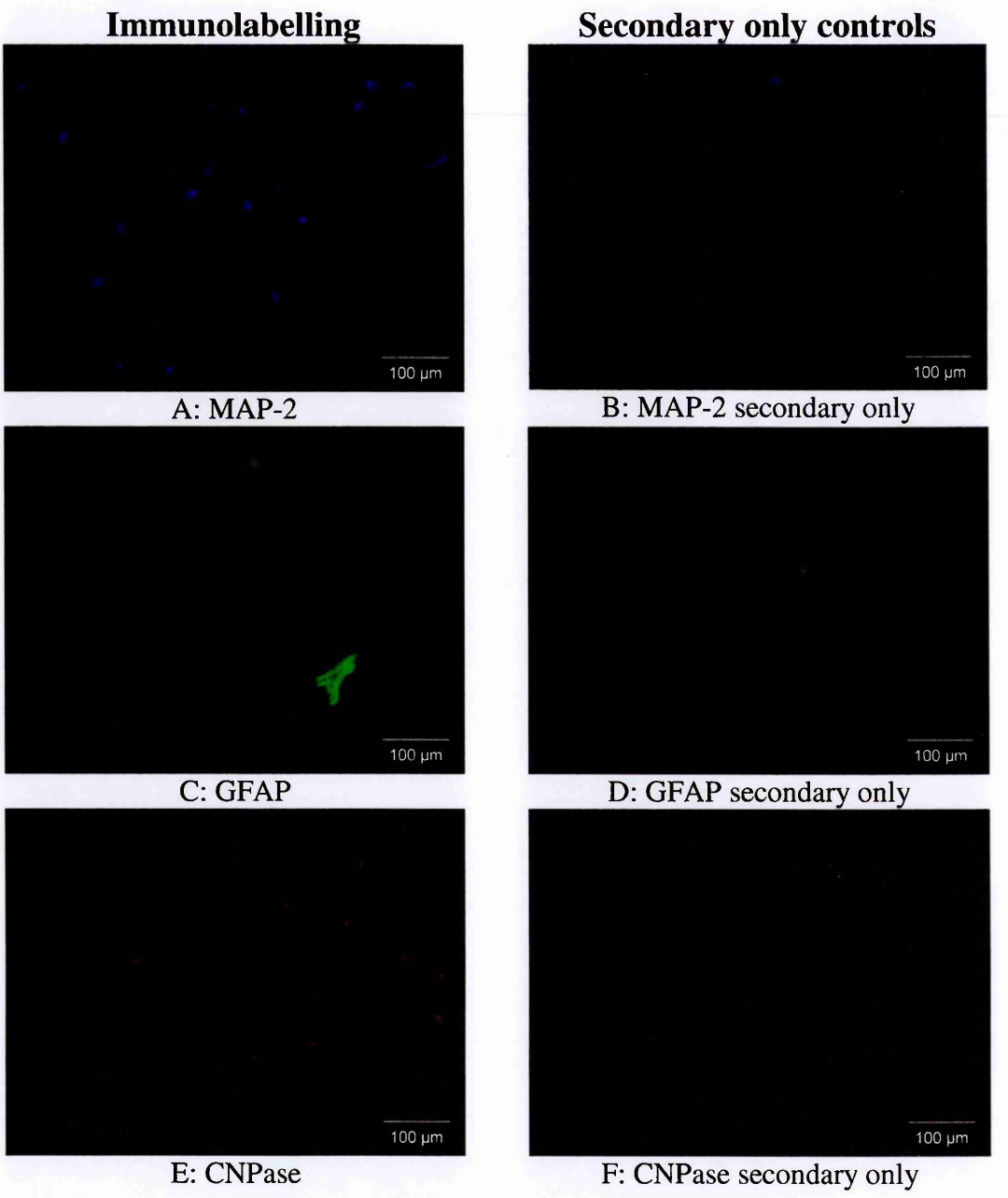


Figure 3-1: Representative images of MAP-2 (A), GFAP (C) and CNPase (E) immunolabelling in cultures from E18 rat hippocampus. Cultures were paraformaldehyde fixed at *DIV*4 and processed for immunocytochemistry. Secondary only controls are also shown for anti-mouse AMCA conjugated (B), anti-rabbit FITC conjugated (D) and anti-mouse Rhodamine conjugated (F) secondary antibodies.

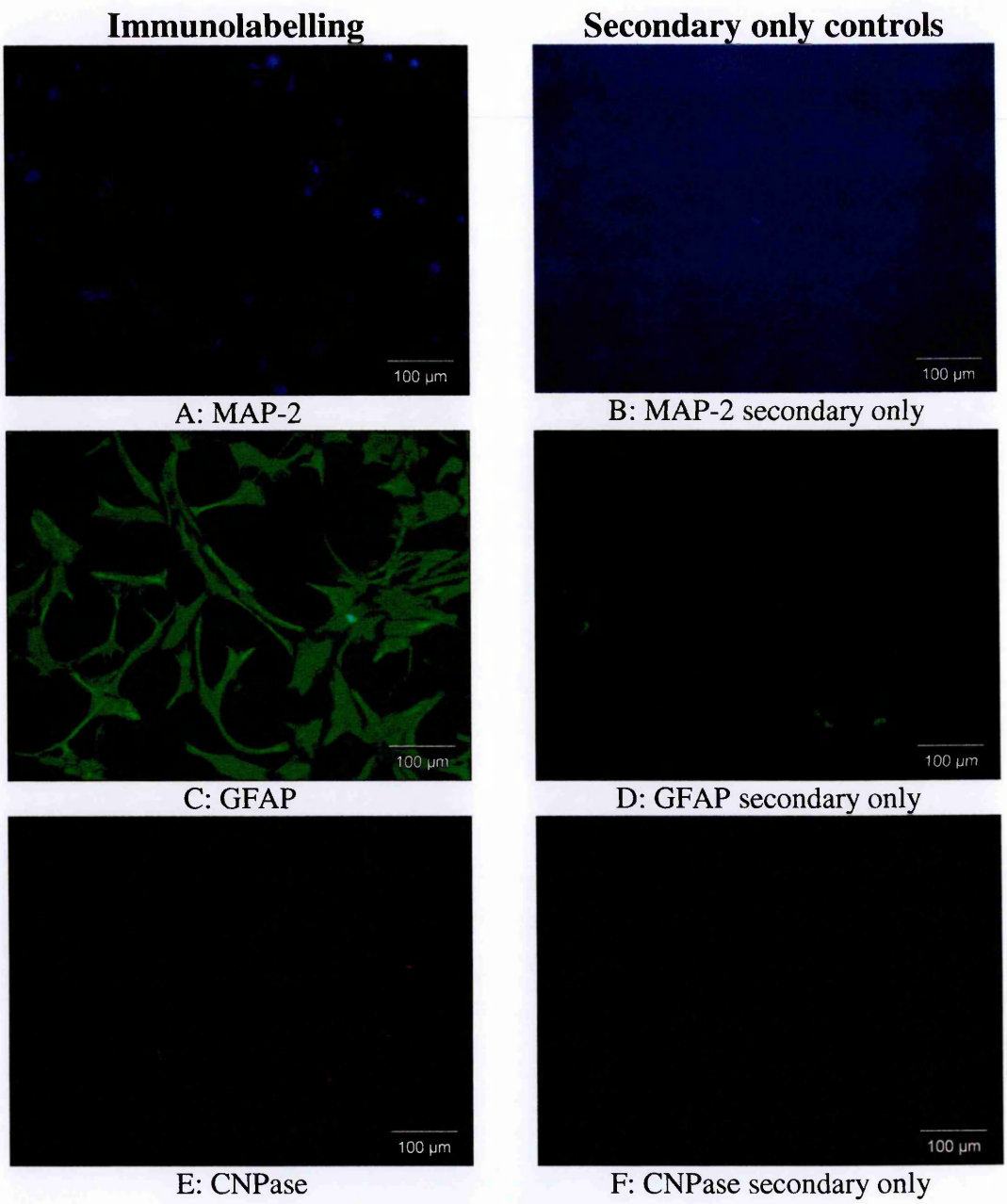


Figure 3-2: Representative images of MAP-2 (A), GFAP (C) and CNPase (E) immunolabelling in cultures from P4 rat hippocampus. Cultures were paraformaldehyde fixed at *DIV*4 and processed for immunocytochemistry. Secondary only controls are also shown for anti-mouse AMCA conjugated (B), anti-rabbit FITC conjugated (D) and anti-mouse Rhodamine conjugated (F) secondary antibodies.

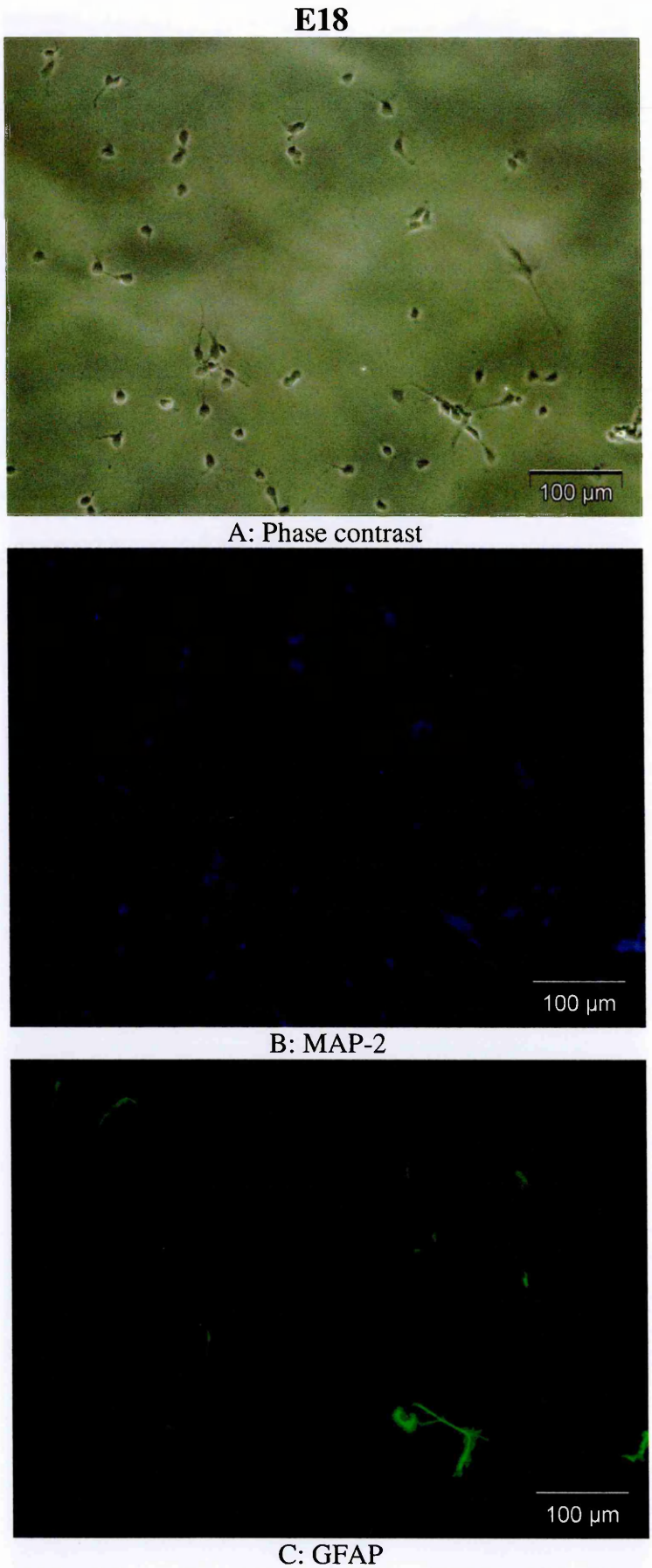
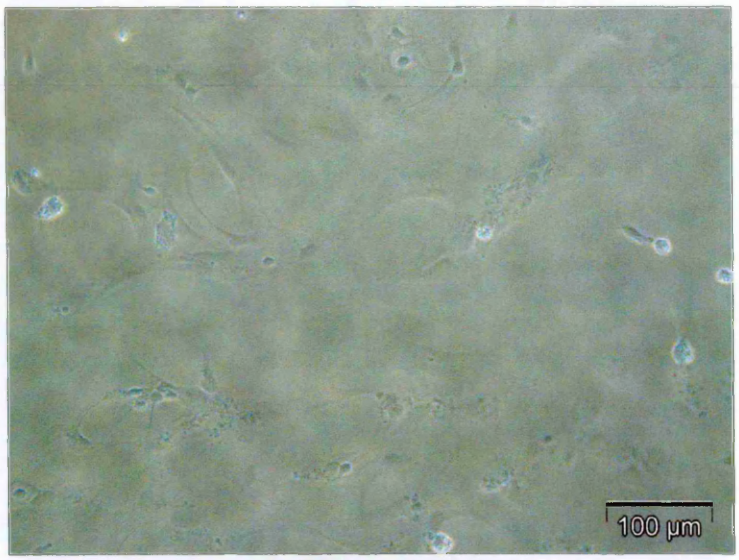
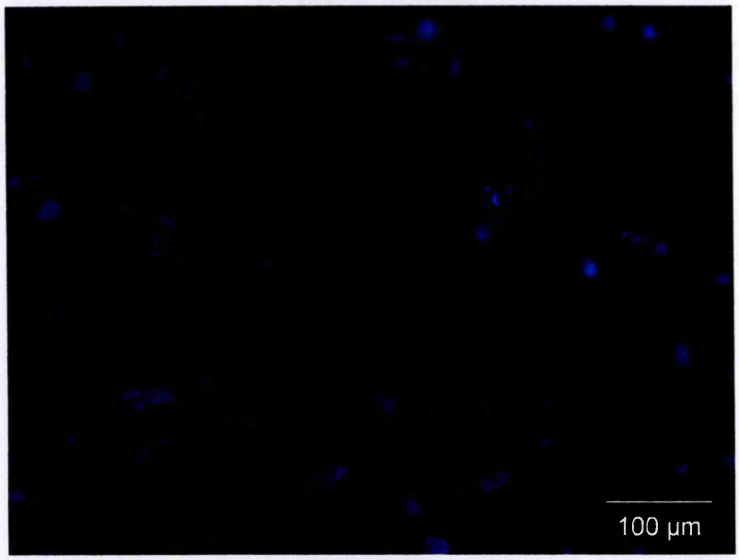


Figure 3-3: Representative phase contrast (A) and the corresponding MAP-2 (B), GFAP (C) immunocytochemistry images of cultures from E18 (A-C) rat hippocampus that were seeded at 40,000 cells/well and paraformaldehyde fixed at *DIV*4 to be processed for immunocytochemistry.

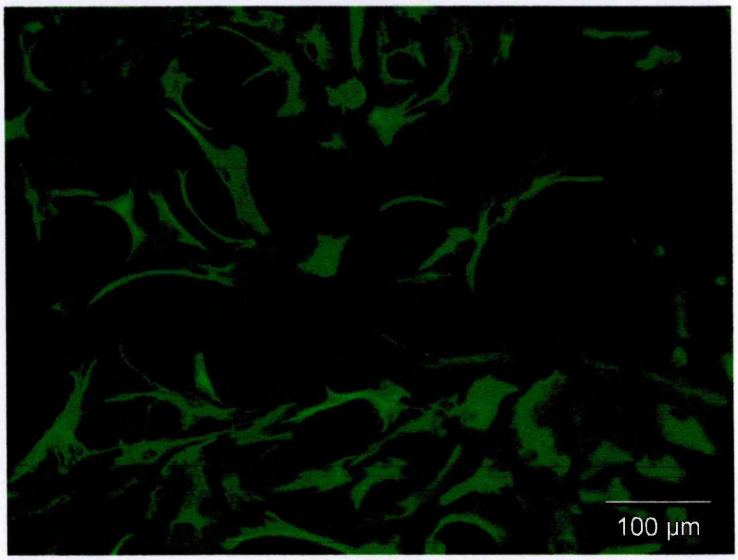
P4



A: Phase contrast



B: MAP-2



C: GFAP

Figure 3-4: Representative phase contrast (A) and the corresponding MAP-2 (B), GFAP (C) immunocytochemistry images of cultures from P4 (A-C) rat hippocampus that were seeded at 40,000 cells/well and paraformaldehyde fixed at *DIV*4 to be processed for immunocytochemistry.

Quantification, carried out using a counting grid on 9mm^2 of each coverslip, as described in Chapter 2, Section 2.8.6, showed that in cultures from E18 rat hippocampus MAP-2 positive neurons outnumbered the GFAP positive astrocytes by approximately 4:1 ($P < 0.001$). The converse was true in cultures from P4 hippocampus and approximately four times as many GFAP positive astrocytes as MAP-2 positive neurons were present in these cultures ($P < 0.001$) (see Figure 3-5). There was also a significant difference between the number of MAP-2 positive neurons at E18 compared to P4 and the number of GFAP positive astrocytes at E18 compared to P4 (see Figure 3-5).

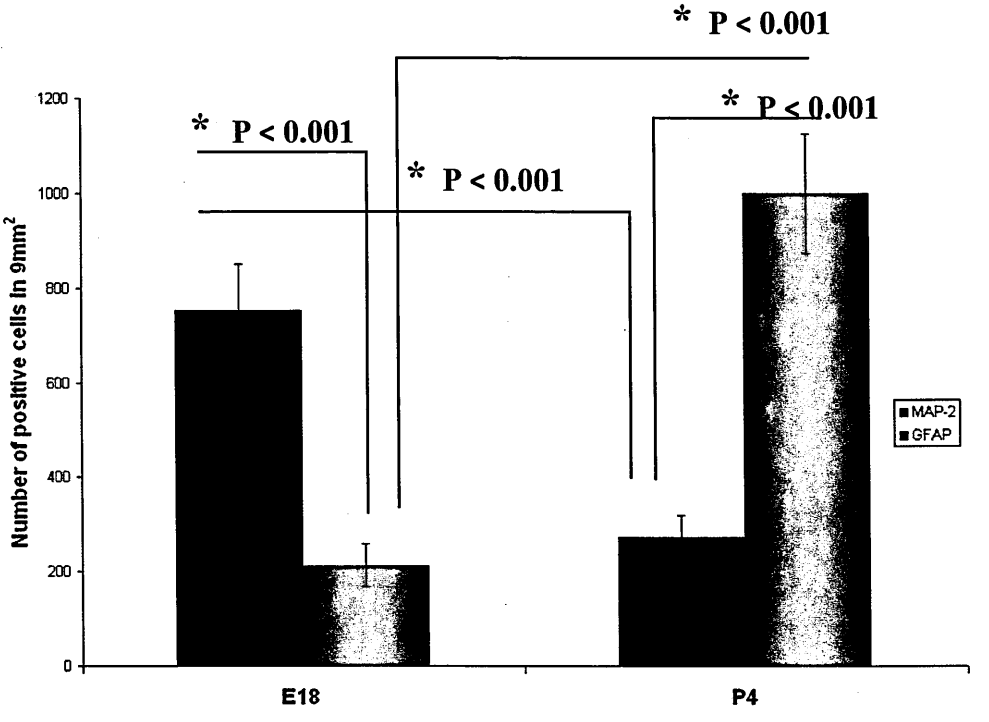


Figure 3-5: Quantification of the relative numbers of MAP-2 and GFAP positive cells in dissociated hippocampal cultures from E18 and P4 rats. There was a significant difference between the number of MAP-2 positive neurons and GFAP positive astrocytes in cultures from both E18 and P4 hippocampi (from one-way ANOVA $P < 0.001$). The number of MAP-2 positive neurons and GFAP astrocytes in cultures from E18 hippocampi were also significantly different to the number of corresponding cells in cultures from P4 hippocampi (from one-way ANOVA $P < 0.001$). n=8 cultures with at least 3 replicates per experiment. Error bars represent standard errors. * represents $P < 0.001$.

3.4.2. The effect of CLN on hippocampal cells in culture

3.4.2.1. The effect of CLN on cell numbers in cultures from E18 hippocampus

In cultures from E18 hippocampi 24 hours of treatment with bovine CLN alone at concentrations ranging between 1ng/ml and 100ng/ml did not have any notable effect on the morphology of cells compared to control cultures and did not cause any notable changes in neurite extension (see Figure 3-6). Quantification showed that 24 hour treatment with CLN at these concentrations had no effect on the number of MAP-2 positive neurons (see Figure 3-7) or GFAP astrocytes (see Figure 3-8).

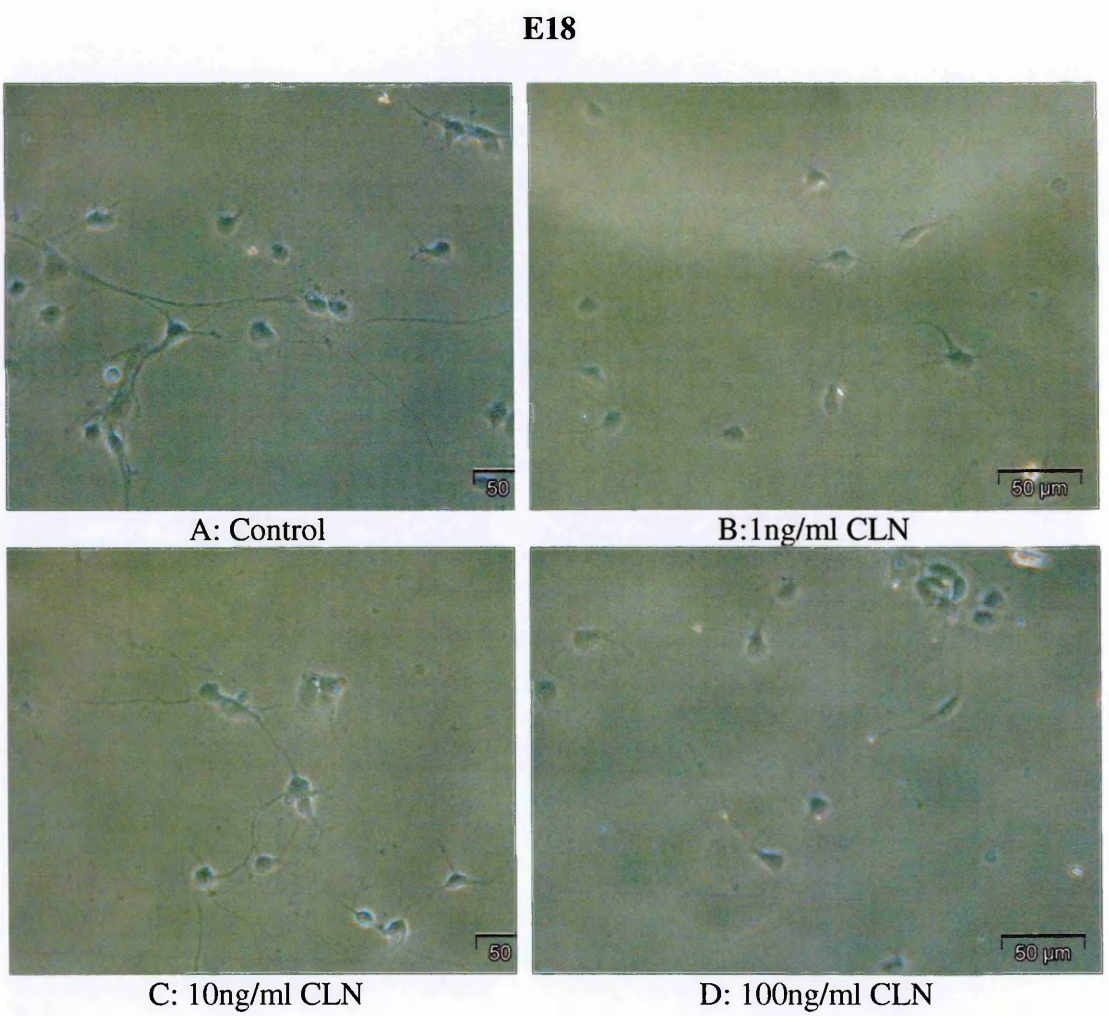


Figure 3-6: Phase contrast images of cultures from E18 hippocampi at *DIV*4 showing that CLN at 1 (B), 10 (C) and 100ng/ml (D) had no effect on cell morphology and neurite outgrowth compared to controls (A).

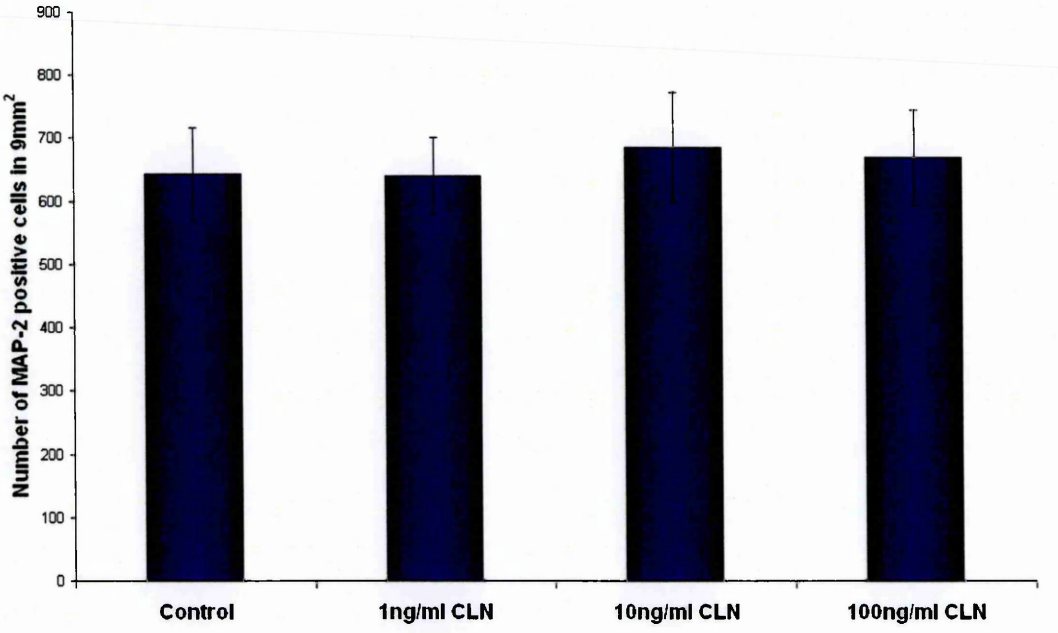


Figure 3-7: Quantification of the numbers of MAP-2 positive neurons in dissociated hippocampal cultures from E18 rats seeded at 40,000 cells/well and treated with CLN showing that there was no effect with CLN ($P > 0.9$ from one-way ANOVA). $n \geq 3$ with at least three replicates per condition. Bars represent standard errors.

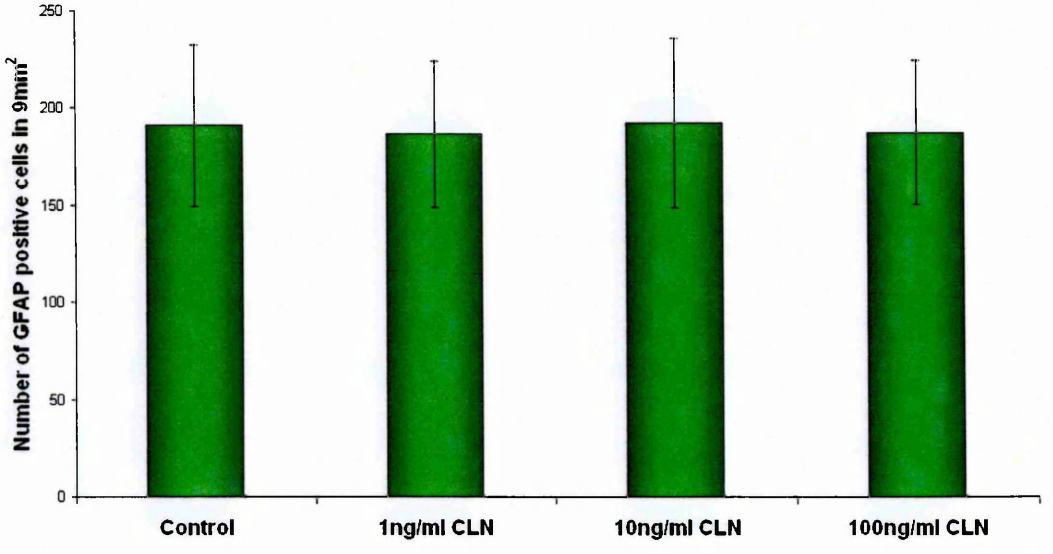


Figure 3-8: Quantification of the numbers of GFAP positive astroytes in dissociated hippocampal cultures from E18 rats seeded at 40,000 cells/well and treated with CLN showing that there was no effect with CLN. ($P > 0.9$ from one-way ANOVA). $n \geq 3$ with at least three replicates per condition. Error bars represent standard errors.

3.4.2.2. The effect of CLN on cell numbers in cultures from P4 hippocampus

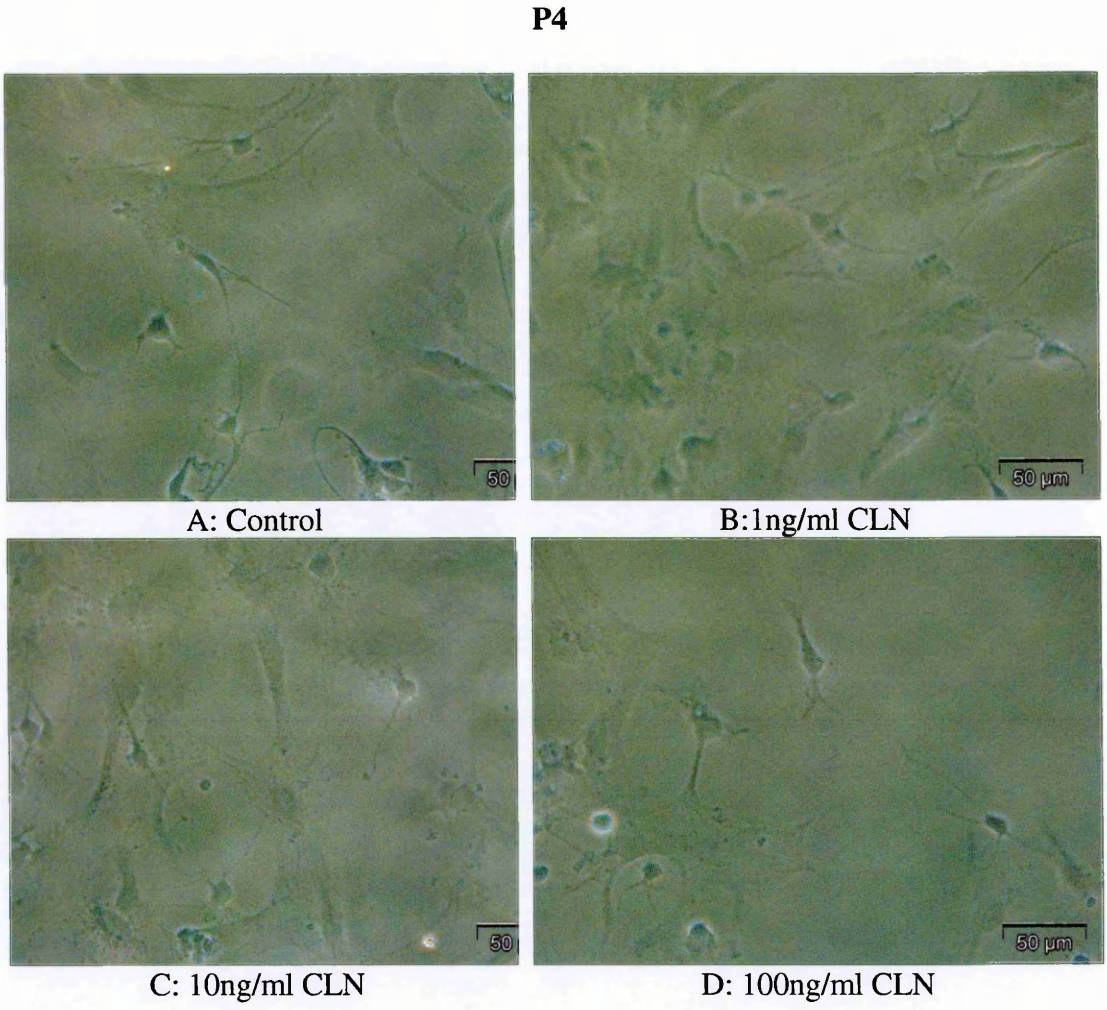


Figure 3-9: Phase contrast images of cultures from P4 hippocampi at *DIV*4 showing that CLN at 1 (B), 10 (C) and 100ng/ml (D) had no effect on cell morphology and neurite outgrowth compared to controls (A).

In cultures from P4 hippocampi, CLN alone at concentrations ranging between 1ng/ml and 100ng/ml also did not have any effect on the morphology of cells compared to control cultures and no notable effect on neurite extension (see Figure 3-9). Quantification of cell numbers confirmed that CLN had no effect on the number of MAP-2 positive neurons (see Figure 3-10) or GFAP positive astrocytes (see Figure 3-11) compared to control cultures.

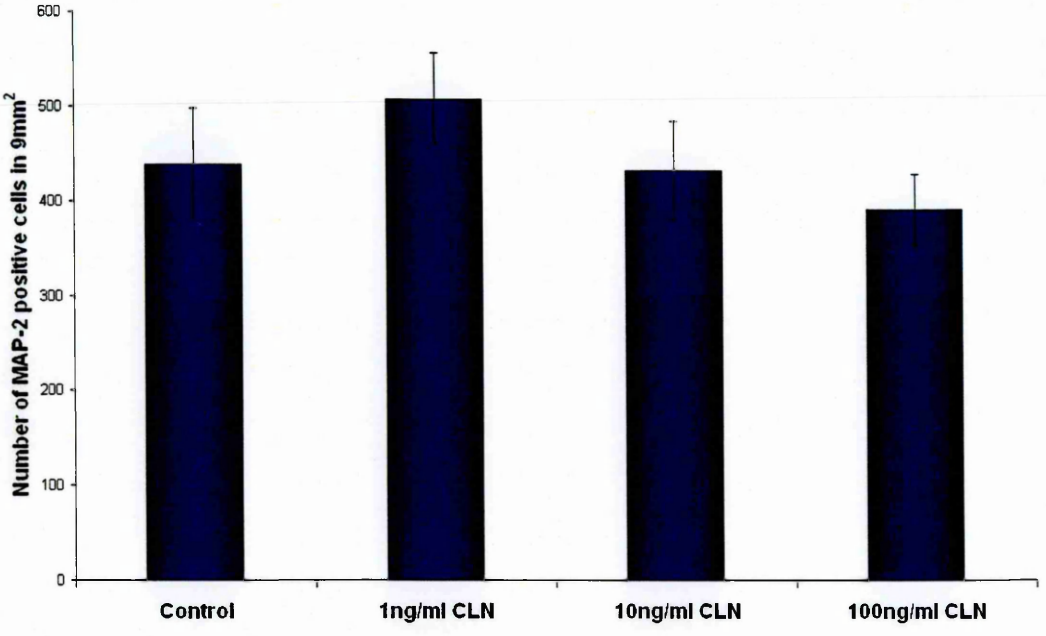


Figure 3-10: Quantification of the numbers of MAP-2 positive neurons in dissociated hippocampal cultures from P4 rats seeded at 40,000 cells/well and treated with CLN showing that there was no effect with CLN on the numbers of these cells present in cultures ($P > 0.9$ from one-way ANOVA). $n \geq 3$ with at least three replicates per condition. Error bars represent standard errors.

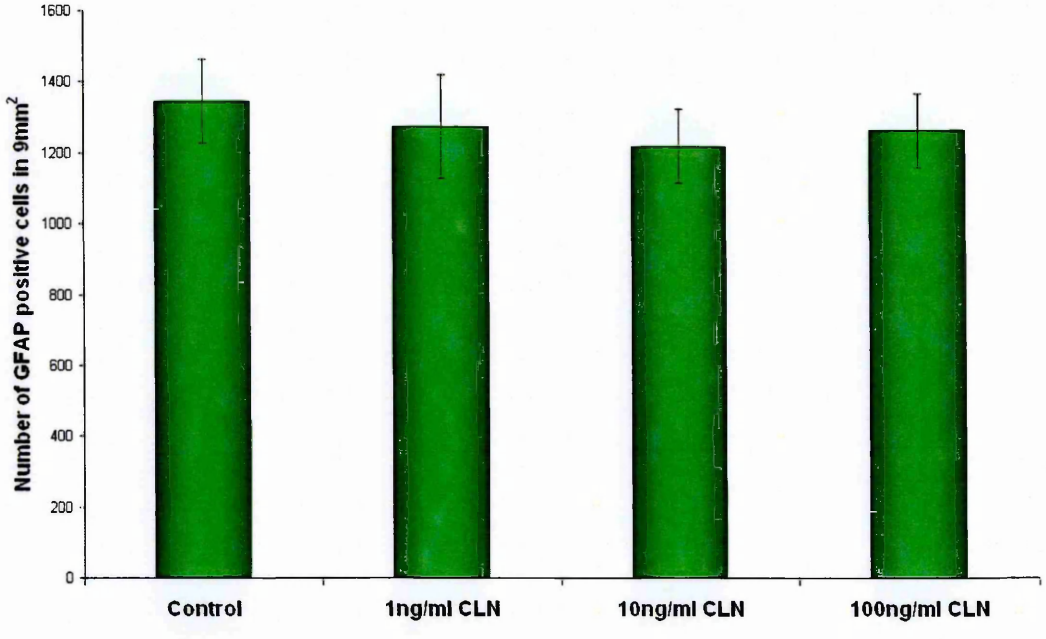


Figure 3-11: Quantification of the numbers of GFAP positive neurons in dissociated hippocampal cultures from P4 rats seeded at 40,000 cells/well and treated with CLN showing that there was no effect with CLN on the numbers of these cells present in cultures ($p > 0.9$ from one-way ANOVA). $n \geq 3$ with at least three replicates per condition. Error bars represent standard errors.

3.4.3. The effect of CLN on hippocampal cell ultrastructure

Investigation of the effects of bovine CLN at the ultrastructural level using EM showed no obvious qualitative changes in any cellular structures, mitochondria, nuclei or cell processes in the cultures with CLN treatment in these conditions, compared to untreated control cultures (see Figures 3-12). There were no necrotic or unusual structures present and none of the cells present appeared to be affected by the CLN treatment over 24 hours. Figure 3-12D shows what may be a synapse although this is not confirmed.

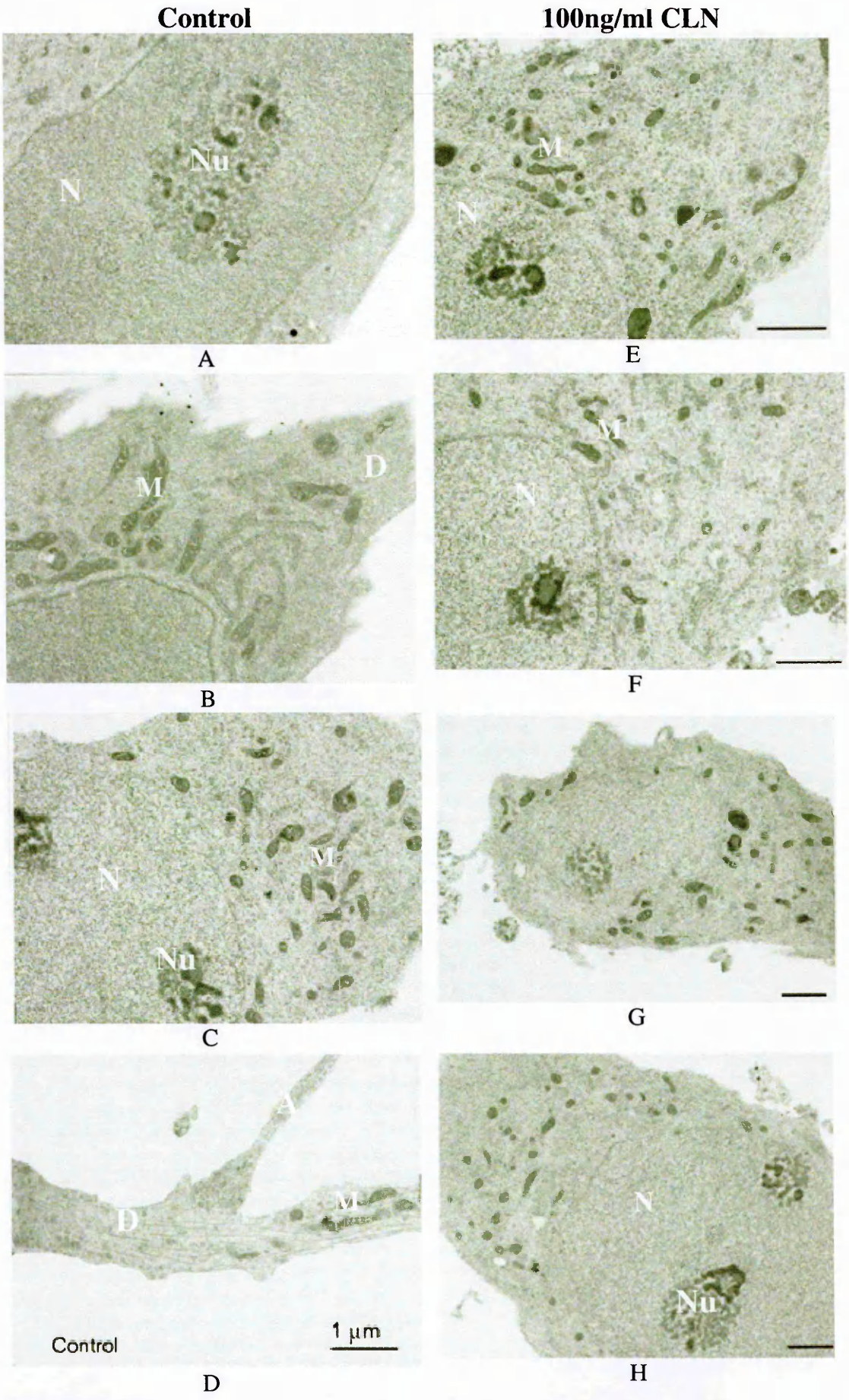


Figure 3-12: EM micrographs of control (A-D) and 100ng/ml bovine CLN treated (E-H) hippocampal cultures from P4 hippocampus fixed at *DIV*7 showing the dendrites (D), axons (A), nuclei (N), nucleoli (Nu) and mitochondria (M).

3.5. Discussion

In any investigation using a culture system as a model for study it is important to fully understand the system being used and the cell types that are present within the cultures in order to be able to completely comprehend the ramifications of any treatment on the cultures and to give the most informed analysis possible of results.

To this end the presence and proportion of neurons, astrocytes, microglia and oligodendroytes in untreated hippocampal cultures was first examined. These experiments showed that the model culture system is a mixed culture containing mainly neurons with astrocytes. The proportions of neurons to astrocytes differed between cultures from E18 hippocampus and those from P4 hippocampus. The greatly increased number of GFAP positive astrocytes relative to MAP-2 positive neurons in cultures from postnatal hippocampus compared to cultures from embryonic hippocampus was expected. This difference in numbers is due to higher numbers of mature astrocytes in postnatal rats (Gasser and Hatten 1990; Eng *et al.* 2000) leading to much higher numbers of GFAP-expressing cells and higher levels of GFAP expression in these cultures and also to the natural death of some neurons during development (Sendtner *et al.* 2000). Inspection of some fields of cultures from embryonic rats under phase contrast and fluorescence confirmed that under phase contrast there were no cells of astrocytic morphology that did not stain positively for GFAP showing that the difference in cell numbers was not due to staining artefacts.

The proportions of cell types that were to be expected in these cultures was detailed in Chapter 1, Section 1.5.2.3. Although microglial proliferation has been observed in organotypic hippocampal cultures from early postnatal rats when the hippocampus has undergone traumatic injury (Laskowski *et al.* 2007) the lack of

microglia in the cultures from E18 hippocampus is in keeping with the observations of Xie *et al.* (2000) showing that in hippocampal cells from E18 rats cultured in basal medium contain very few microglia. These authors also found very similar proportions of MAP-2 and GFAP positive cells to those observed in this study in cultures from E18 rats cultured in basal medium with B27 as they were here (Xie *et al.* 2000).

In cultures from both E18 and P4 hippocampus CLN ranging between 1ng/ml and 100ng/ml had no trophic effect. EM data indicated that there were no ultrastructural changes in cultures from P4 hippocampus treated with bovine CLN at 100ng/ml for 24 hours.

This work demonstrated that the hippocampal cultures as used in these experiments contain neurons and astrocytes and that the proportions of these cell types changes at different stages of development. Moreover it indicates that bovine CLN has no adverse effects on primary hippocampal cells in these conditions.

The higher proportion of neurons to glia and greater proportion of neurons in the cultures from E18 rats seen in these initial experiments compared to those from P4 rats led to the decision to continue using cultures from E18 hippocampus for the subsequent work.

Chapter 4

The effects of bovine CLN on beta-amyloid and menadione-induced toxicity in cells in culture

4.1. Aims

The aim of these experiments was to investigate the possible protective effect of bovine CLN against menadione-induced, oxidative stress-mediated, toxicity, and $A\beta_{1-42}$-induced toxicity on primary hippocampal cells, cultured as described in Chapter 3, and the B50 cell line in culture. The rationale for using B50 cells as a model system is that it is a rat neuronal cell line and therefore is a good cell line comparison to the primary rat hippocampal cells and it has previously been used for toxicity studies as discussed in Chapter 2, Section 2.4.

In order to observe any changes in the morphology or number of cells in these cultures in the presence of $A\beta_{1-42}$ or menadione and bovine CLN, MAP-2 positive neurons and GFAP positive astrocytes were studied. Dissociated hippocampal cultures were treated with $A\beta_{1-42}$, menadione and bovine CLN and double immunolabelled for MAP-2 and GFAP. The ability of bovine CLN to protect these cells against the toxicity of menadione and $A\beta_{1-42}$ was investigated.

An MTS assay was carried out to assess changes in cell survival in the B50 cell line upon treatment with menadione and bovine CLN.

4.2. Introduction

4.2.1. Models of oxidative stress *in vitro*

H_2O_2 treatment has commonly been used as a method of inducing oxidative stress *in vitro*. Treatment with H_2O_2 leads directly to increased ROS production and oxidative stress-induced damage in many cell types in culture, including rat neuronal cell lines (Iwata *et al.* 1998; Miyazaki *et al.* 1999) and rat hippocampal slice cultures (Avshalumov and Rice 2002). Moreover, H_2O_2 has been extensively used on primary hippocampal neurons in culture as a model of cell death in response to oxidative stress (Zhang *et al.* 2001; Avshalumov and Rice 2002). However, H_2O_2 can be quite unstable over time and is sensitive to exposure to light leading to reduction in its activity.

A regularly used alternative method for creating a model of oxidative stress in cell culture is to treat cells with the compound menadione. Menadione is a semi-quinone (see Figure 4-1) and leads to the production of ROS including H_2O_2 and superoxide as it is reduced from the semi-quinone to the quinone. Menadione may then be re-oxidised and in this manner a redox cycle can be continued leading to the accumulation of ROS which can cause significant cellular damage and cell death.

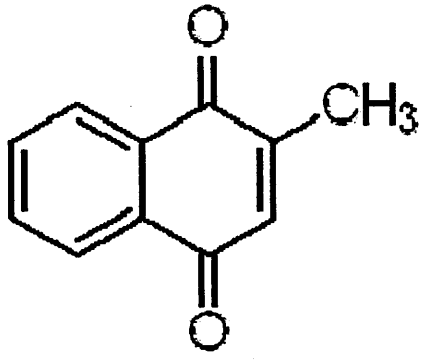


Figure 4-1: The structure of menadione (from www.sigmaaldrich.com).

4.2.2. CLN and protection against oxidative stress-induced toxicity

As described in detail in Chapter 1, Section 1.5.3 the antioxidant effects of CLN have been extensively studied and there has been evidence for such anti-oxidant effects in several cell types (Boldogh *et al.* 2003; Bacsi *et al.* 2005; Bacsi *et al.* 2006; Bacsi *et al.* 2007). However, no research has yet been done to analyse the effects of CLN on the survival of cells in culture subjected to oxidative stress-induced toxicity.

The first part of this work therefore aimed to assess the protective effects of CLN against oxidative stress using menadione-induced neurotoxicity in primary hippocampal cultures and the B50 cell line. Menadione was chosen as the oxidative stress inducer in these experiments because it has been widely used in a variety of cell types such as hepatocytes (Thor *et al.* 1982) and myenteric plexus neurons (Thrasivoulou *et al.* 2006) and is not as sensitive to temperature and time as H_2O_2. Moreover, it has previously been found by Adamec *et al.* (2000) that treatment with 10μM menadione for 24 hours will lead to significant levels of cell death in primary hippocampal cultures.

4.2.3. Beta-amyloid-induced toxicity in hippocampal cells in culture

As discussed in Chapter 1, Section 1.1.2 the current leading theory of AD pathology is the amyloid hypothesis. Prevention of Aβ-induced toxicity is therefore seen as the key to preventing AD progression.

The hippocampus is known to be highly vulnerable to AD-induced cell death and this damage is a major cause of the disease symptoms of memory loss and cognitive decline (Rodriguez *et al.* 2008). Therefore the effects of Aβ on hippocampal cells in culture have been extensively studied (Pike *et al.* 1991; Liu *et al.* 2001; Kelly and Ferreira 2007; Resende *et al.* 2007). The importance of the

aggregation state of Aβ on the toxicity has been discussed in Chapter 1, Section 1.1.2. Several authors have demonstrated the aggregation-dependent toxicity of $A\beta_{1-40}$ (Harris *et al.* 1995; Liu *et al.* 2005; Resende *et al.* 2007), $A\beta_{1-42}$ (Pike *et al.* 1991; Lorenzo and Yankner 1994; Puttfarcken *et al.* 1996; Zou *et al.* 2003; Liu *et al.* 2005) and $A\beta_{25-35}$ (Forloni *et al.* 1993; Takadera *et al.* 1993; Resende *et al.* 2007) on hippocampal cultures from embryonic rats.

As well as the aggregation state and form of Aβ used, the age of the culture when treated may also be important to the level of toxicity achieved. $A\beta_{1-42}$ has been found to be toxic when hippocampal cells were treated on *DIV*1 for 48 hours but it has been shown that $A\beta_{1-40}$ has a higher level of toxicity on hippocampal cells that have been in culture for 3 days or more than on 1 or 2 day old cultures (Whitson and Appel 1995). Furthermore, Yankner *et al.* (1990) showed that $A\beta_{1-40}$ may even be neurotrophic to new hippocampal cultures and only become toxic on mature cultures of *DIV*3 or more.

4.2.4. CLN and protection against beta-amyloid induced toxicity

CLN has been demonstrated to be beneficial to AD patients and halt disease progression but there has been very little work to directly analyse the effects of CLN on Aβ-induced cell death in cultured cells. The focus has been on the effects of CLN in patients (Leszek *et al.* 1999; Leszek *et al.* 2002), on spatial memory in rats (Popik *et al.* 1999; Popik 2001; Popik *et al.* 2001) and on the prevention of oxidative stress induction (Boldogh *et al.* 2003; Bacsi *et al.* 2006; Bacsi *et al.* 2007). There has only been one study investigating the effects of CLN on cell survival after Aβ-induced toxicity. Schuster *et al* (2005) showed that pre-incubation and co-administration of

CLN, at low concentrations, with $A\beta_{1-40}$ could protect SH-SY5Y human neuroblastoma cells against $A\beta_{1-40}$ induced toxicity.

The second part of this work replicated the study by Shuster *et al.* (2005), that was described above using primary hippocampal cells in order to investigate whether the protective effect of CLN seen in the cell line also occurs in primary hippocampal cells in culture. $A\beta_{1-42}$ was used instead of $A\beta_{1-40}$ for two reasons; firstly because of the reported increased toxicity of $A\beta_{1-42}$ and secondly because $A\beta_{1-42}$ can be dissolved in distilled water and PBS whereas $A\beta_{1-40}$ has to be dissolved in acid (Biosource product data sheets for 03-136 and 03-112) which may interfere with other effects on the cultures.

In addition to the effects of astrocytes on neuronal survival as discussed in Chapter 3, Section 3.2.2 astrocytic plaques have been found to be involved in the development of the toxicity in AD (Nagele *et al.* 2004). It is entirely feasible that that any effects of CLN on neurons may be due to changes in the astrocytes within the cultures. It was therefore also considered of great importance as part of these experiments to look at the effects of $A\beta_{1-42}$, bovine CLN and menadione on GFAP positive astrocytes as well as effects on neurotoxicity.

4.3. Methods

4.3.1. Immunocytochemistry

4.3.1.1. Preparation of cell cultures

The method for the culture of dissociated hippocampal cells was as described in Chapter 2; Section 2.3. For immunocytochemistry the cells were plated in 12 well plates with at least three replicates per condition at 80,000 cells/well to allow cell-cell interactions or 20,000 cells/well to allow clearer visualisation of any changes in neurite extension.

4.3.1.2. Menadione treatment

Bovine CLN and menadione were prepared as described in Chapter 2, Section 2.5 and 2.6 respectively. On *DIV*3 cultures were treated with 10μM menadione alone in 0.01% ethanol, 100ng/ml or 1μg/ml bovine CLN alone or menadione with bovine CLN for 24 hours. Control cultures were treated with 0.01% ethanol in the culture media and bovine CLN was diluted in 0.01% ethanol in culture media to ensure that any changes observed were not due to the presence or absence of ethanol used to dissolve the menadione.

4.3.1.3. Beta-amyloid treatment

Bovine CLN was prepared as described in Chapter 2, Section 2.5 and $A\beta_{1-42}$ was prepared as described in Chapter 2, Section 2.7. On *DIV*3 cultures were treated with 25μM $A\beta_{1-42}$ alone, 5μg/ml bovine CLN alone or $A\beta_{1-42}$ with bovine CLN for 48 hours. Control cultures were treated with an equal amount of PBS to that in which the $A\beta_{1-42}$ is diluted and bovine CLN given alone was diluted in PBS. 25μM $A\beta_{1-42}$

was chosen for this experiment because this is a concentration that has previously been shown to cause toxicity in these cells (Woods *et al.* 1995) and the work carried out by Schuster *et al* (2005) used 25μM $A\beta_{1-40}$.

4.3.1.4. Immunocytochemistry and quantification

Cells were fixed at *DIV*5 in 4% paraformaldehyde and immunocytochemistry for GFAP and MAP-2 was carried out as described in Chapter 2, Section 2.8. Positively stained cells were then counted as described in Chapter 2, Section 2.8.6.

4.3.2. MTS cytotoxicity assay in the B50 cell line

B50 cells were plated as described in Chapter 2, Section 2.10. Cells were treated on *DIV*1 with 10ng/ml, 500ng/ml, 2μg/ml or 10μg/ml bovine CLN 24 hours prior to the addition of 10 or 20μM menadione or at the time of menadione treatment for a further 24 hours. Control cultures and CLN treated cultures were treated with 0.01 or 0.02% ethanol to ensure that any changes observed were not due to the ethanol used to dissolve the menadione. The MTS assay was then carried out as detailed in Chapter 2, Section 2.10.

4.4. Results

4.4.1. The effect of CLN against menadione-induced toxicity

4.4.1.1. The effect of menadione and CLN on cell morphology and numbers in primary hippocampal cultures

For this experiment cultures were plated at a higher density (80,000 cells/well) than the cultures described in Chapter 3 to allow for more cell-cell interaction because this interaction may affect cell survival.

Bovine CLN alone at 100ng/ml had no marked effect on the morphology of MAP-2 positive neurons in primary hippocampal cultures at a density of 80,000 cells/well (see Figure 4-3A and B).

10μM menadione alone caused a reduction in the number of MAP-2 positive neurons compared to control cultures (see Figure 4-3A and C) and quantification showed this to be a significant reduction of 52±12% ($P < 0.001$ see Figure 4-5). Administration of 100ng/ml bovine CLN with 10μM menadione caused a small increase in the number of MAP-2 positive neurons compared to menadione treatment alone (see Figure 4-3C and D) which upon quantification was found to be non-significant ($P = 0.274$ see Figure 4-5).

However, neither 100ng/ml CLN nor 10μM menadione given independently or together caused a visible change in the number of GFAP positive astrocytes present in the cultures (see Figure 4-4) and this was confirmed by quantification data (see Figure 4-6). Menadione treatment therefore caused a 50% decrease in the neuron/astrocyte ratio but bovine CLN alone at 100ng/ml did not affect the neuron/astrocyte ratio.

Phase contrast: cultures at 80,000 cells/well

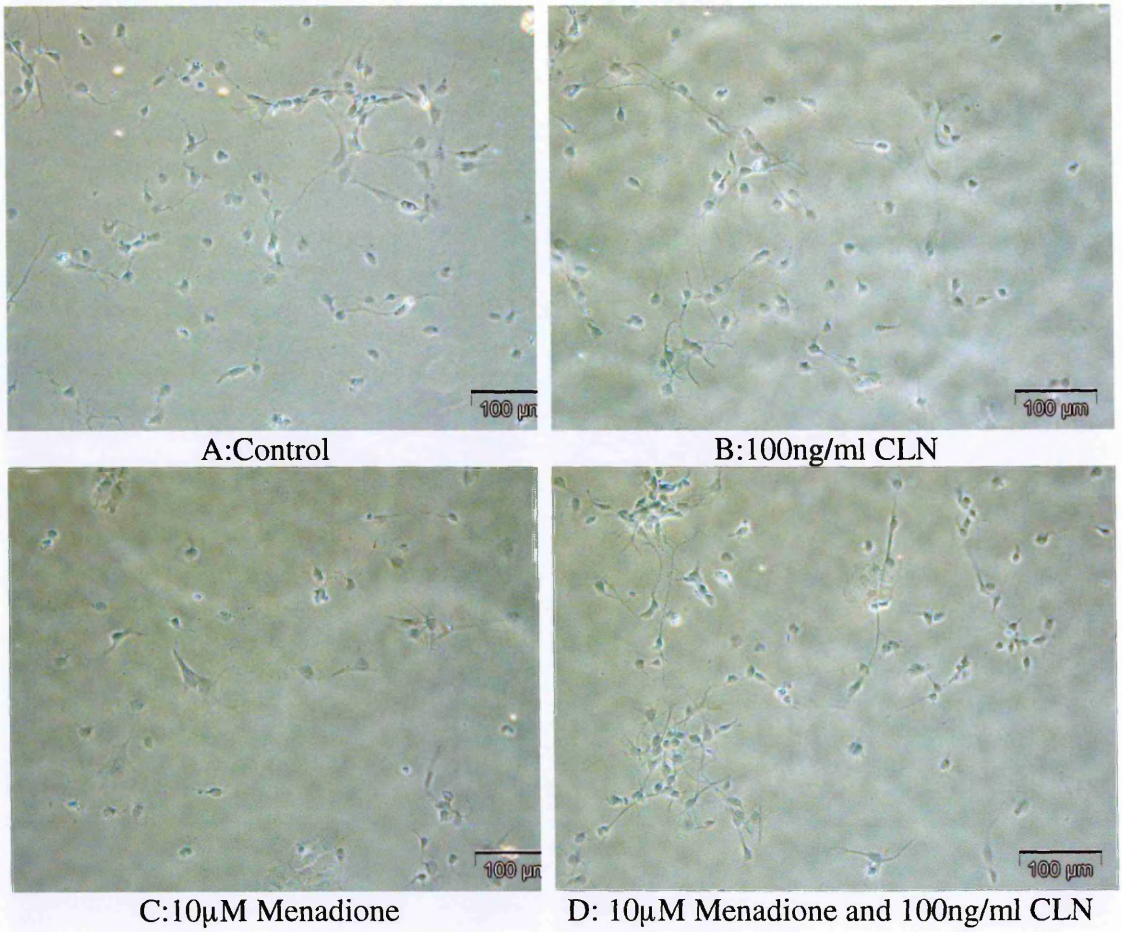


Figure 4-2: Phase contrast images of 80,000 cells/well cultures from E18 hippocampi treated with 0.01% alcohol in culture media (control. A), 100ng/ml CLN (B), 10µM menadione (C) or CLN with menadione (D) for 24 hours. Control cultures (A) contained rounded cells with processes along with some flat cells. CLN treatment (B) did not appear to affect the total cell number compared to controls. However, menadione treatment (C) led to a reduction in the total number of cells present in the culture compared to control.

MAP-2 positive neurons: cultures at 80,000 cells/well

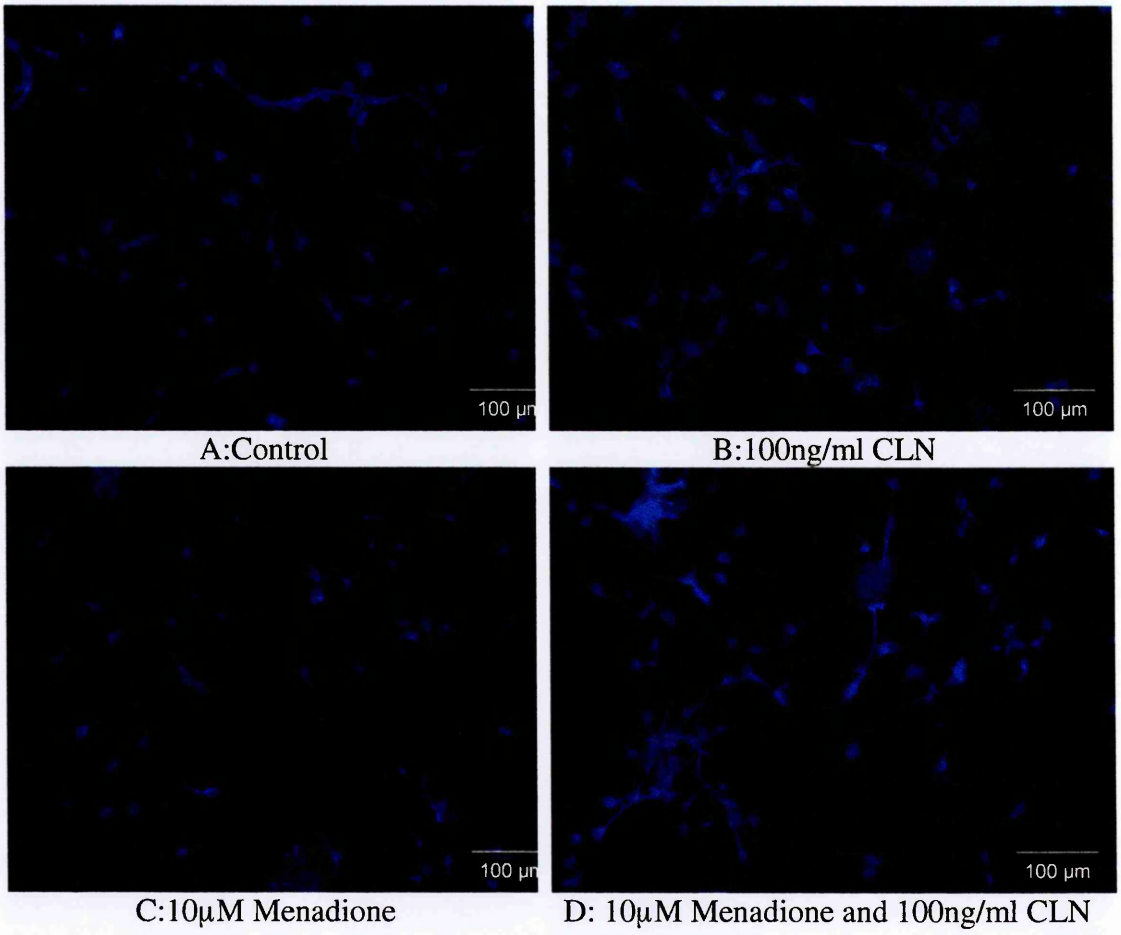


Figure 4-3: Images of MAP-2 immunolabelling on 80,000 cells/well cultures from E18 hippocampi treated with 0.01% alcohol in culture media (control. A), 100ng/ml CLN (B), 10µM menadione (C) or CLN with menadione (D) for 24 hours. Control cultures (A) had rounded MAP-2 positive neurons with processes. The morphology of MAP-2 positive neurons in cultures treated with 100g/ml CLN (B) did not differ from control cultures. However, treatment with menadione (C) led to a change in morphology and a reduction in the number of MAP-2 positive neurons compared to control cultures. This effect was only slightly reversed when CLN was given along with menadione (D).

GFAP positive astrocytes: cultures at 80,000 cells/well

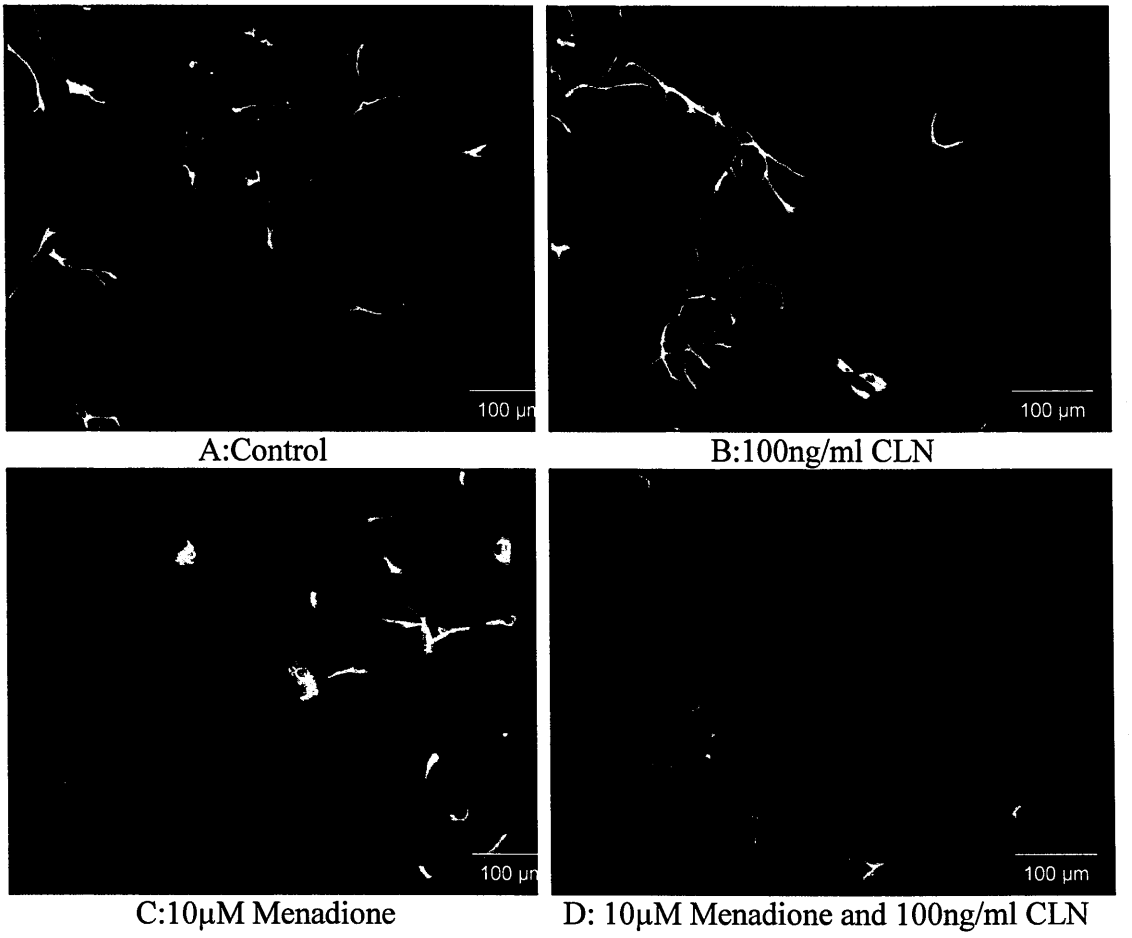


Figure 4-4: Images of GFAP immunolabelling on 80,000 cells/well cultures from E18 hippocampi treated with 0.01% alcohol in culture media (control. A), 100ng/ml CLN (B), 10µM menadione (C) or CLN with menadione (D) for 24 hours. Control cultures (A) contained reasonably small numbers of GFAP positive astrocytes. The number and morphology of GFAP positive astrocytes was not affected by CLN treatment (B) and these cells were relatively unaffected by menadione treatment (C) compared to MAP-2 positive neurons (Figure 4-3C).

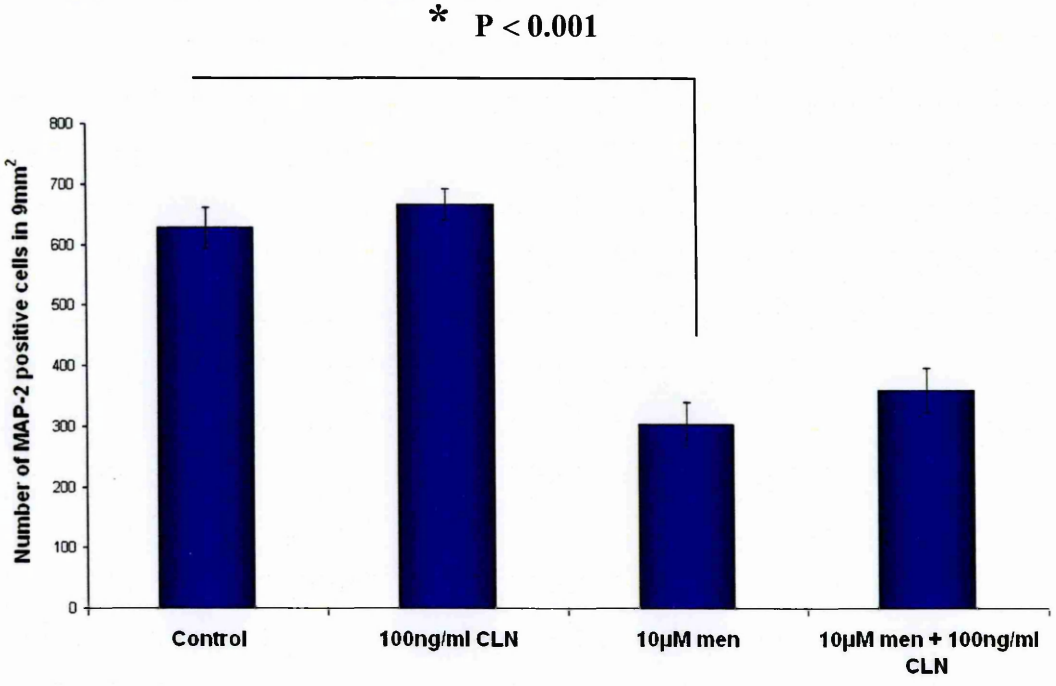


Figure 4-5: Quantification of the number of MAP-2 positive neurons present in primary E18 rat hippocampal cultures at 80,000 cells/well under treatment conditions with 100ng/ml CLN, 10µM menadione or both together. This shows that CLN had no effect on the number of MAP-2 positive cells present in the cultures but menadione treatment caused a 52±12% reduction in the number of MAP-2 positive cells present (from one-way ANOVA $P < 0.001$). n=3 with at least 3 replicates per condition per experiment. Bars represent standard errors. * represents $P < 0.001$.

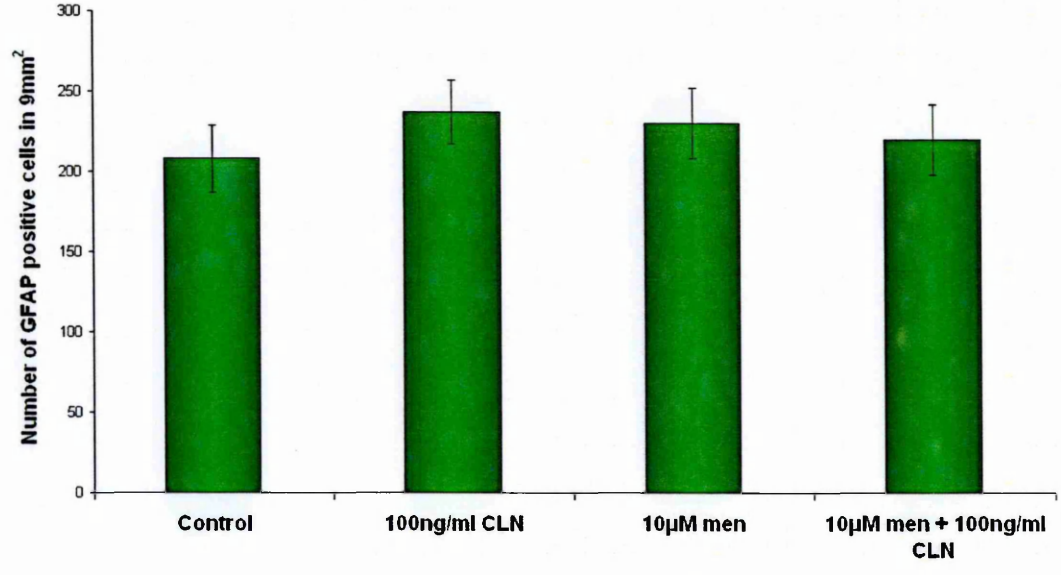


Figure 4-6: Quantification of the number of GFAP positive astrocytes present in primary E18 rat hippocampal cultures at 80,000 cells/well under treatment conditions with 100ng/ml CLN, 10µM menadione or both together. None of the treatment conditions had any notable effect on the number of GFAP positive astrocytes present in the cultures. n=3 with at least 3 replicates per condition per experiment. Bars represent standard errors.

For subsequent experiments cultures were set up at 20,000 cells/well rather than 80,000 cells/well in order to allow better visualisation of any changes in neurite extension and length.

100ng/ml bovine CLN had no visible effect on the morphology of MAP-2 positive or GFAP positive cells in these lower density cultures compared to controls (see Figures 4-8 and 4-9) but caused an increase in MAP-2 positive neurons which upon quantification was found not to be significant ($P = 0.207$. see Figure 4-10). Closer inspection showed that this increase in the number of MAP-2 positive neurons did not appear to be accompanied by an increase in neurite extension (see Figure 4-12).

The toxic effect of menadione was more pronounced in these lower density cultures and 10μM menadione caused a large reduction in the number of MAP-2 positive neurons compared to control cultures (see Figure 4-8A and C) with a small decrease in GFAP positive astrocyte numbers (see Figure 4-9A and C). Quantification showed that the menadione-induced reduction in the number of MAP-2 positive neurons was 90±22% compared to controls and that 100ng/ml bovine CLN given with menadione led to a doubling of the number of MAP-2 positive neurons compared to menadione treatment alone, however this did not quite reach significance ($P = 0.073$ see Figures 4-10). The small menadione-induced decrease in the number of GFAP positive astrocytes was not significant ($P = 0.329$. see Figure 4-11).

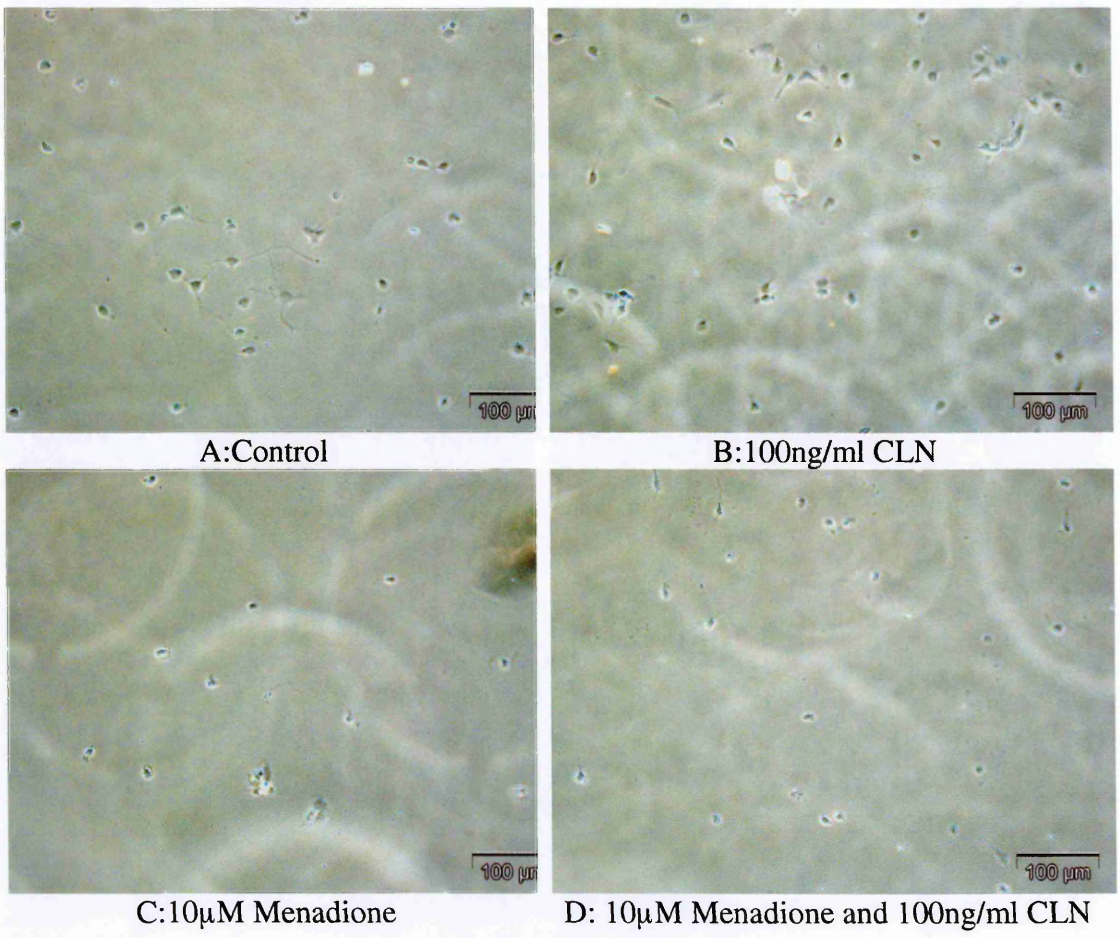


Figure 4-7: Phase contrast images of 20,000 cells/well cultures from E18 hippocampi treated with 0.01% alcohol in culture media (control. A), 100ng/ml CLN (B), 10µM menadione (C) or CLN with menadione (D) for 24 hours. Control cultures (A) contained rounded cells with processes along with some flat cells. CLN treatment (B) did not appear to affect the total cell number compared to controls. However, menadione treatment (C) led to a reduction in the total number of cells present in the culture compared to control.

MAP-2 positive neurons: cultures at 20,000 cells/well

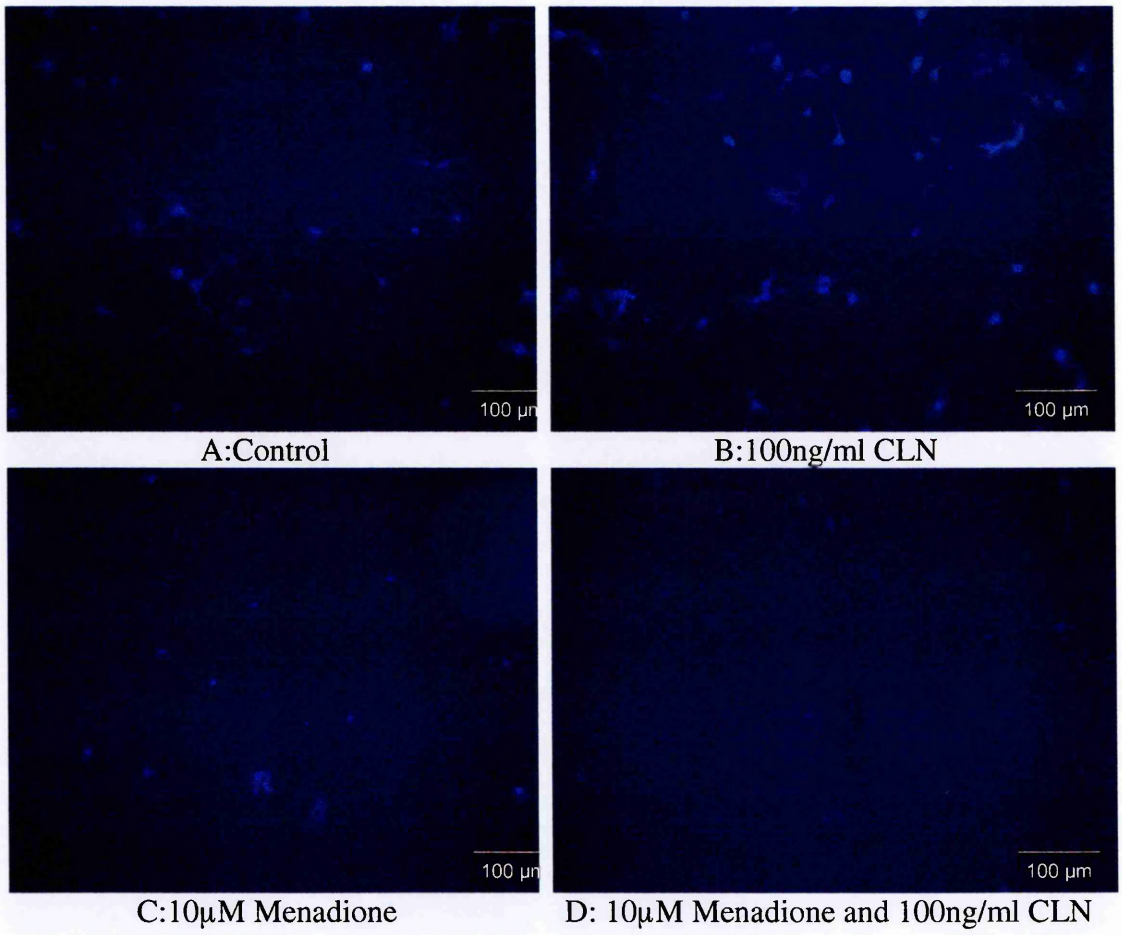


Figure 4-8: Images of MAP-2 immunolabelling on 20,000 cells/well cultures from E18 hippocampi treated with 0.01% alcohol in culture media (control. A), 100ng/ml CLN (B), 10µM menadione (C) or CLN with menadione (D) for 24 hours. Control cultures (A) had rounded MAP-2 positive neurons with processes. The morphology of MAP-2 positive neurons in cultures treated with 100g/ml CLN (B) did not differ from control cultures. However, treatment with menadione (C) led to a change in morphology and a reduction in the number of MAP-2 positive neurons compared to control cultures. This effect was only slightly reversed when CLN was given along with menadione (D).

GFAP positive astrocytes: cultures at 20,000 cells/well

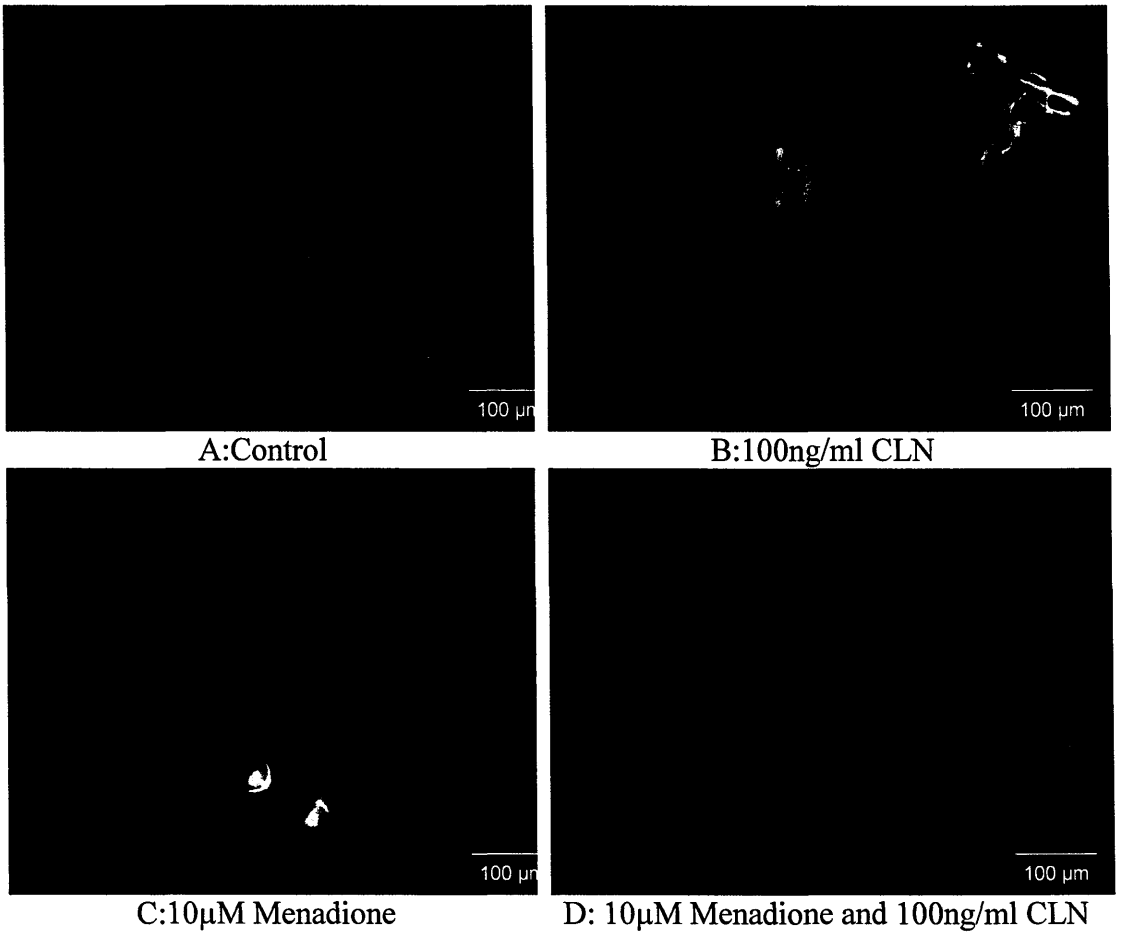


Figure 4-9: Images of GFAP immunolabelling on 20,000 cells/well cultures from E18 hippocampi treated with 0.01% alcohol in culture media (control. A), 100ng/ml CLN (B), 10µM menadione (C) or CLN with menadione (D) for 24 hours. Control cultures (A) contained reasonably small numbers of GFAP positive astrocytes. The number and morphology of GFAP positive astrocytes was not affected by CLN treatment (B) and these cells were only slightly affected by menadione treatment (C) compared to control cultures.

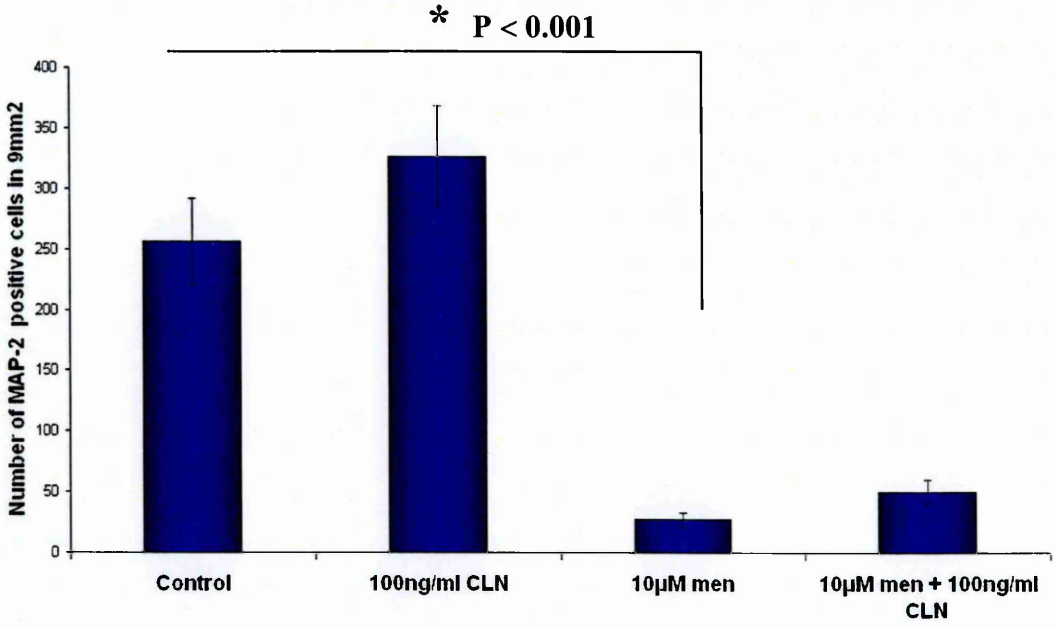


Figure 4-10: Quantification of the number of MAP-2 positive cells present in primary E18 rat hippocampal cultures at 20,000 cells/well under treatment conditions with 100ng/ml CLN, 10µM menadione or both together. 100ng/ml CLN alone had a small trophic effect on the MAP-2 positive neurons in these cultures. Menadione treatment caused 90±22% reduction in the number of MAP-2 positive neurons present in the cultures (from one-way ANOVA $P < 0.001$). a n=3 with at least 3 replicates per condition per experiment. Errors bars represent standard errors. * represents $P < 0.001$.

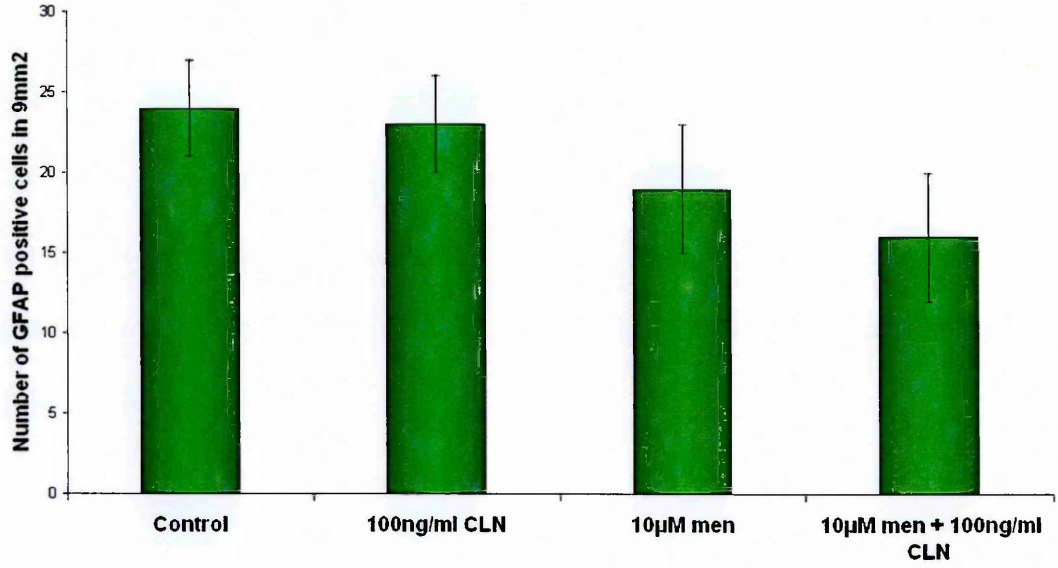
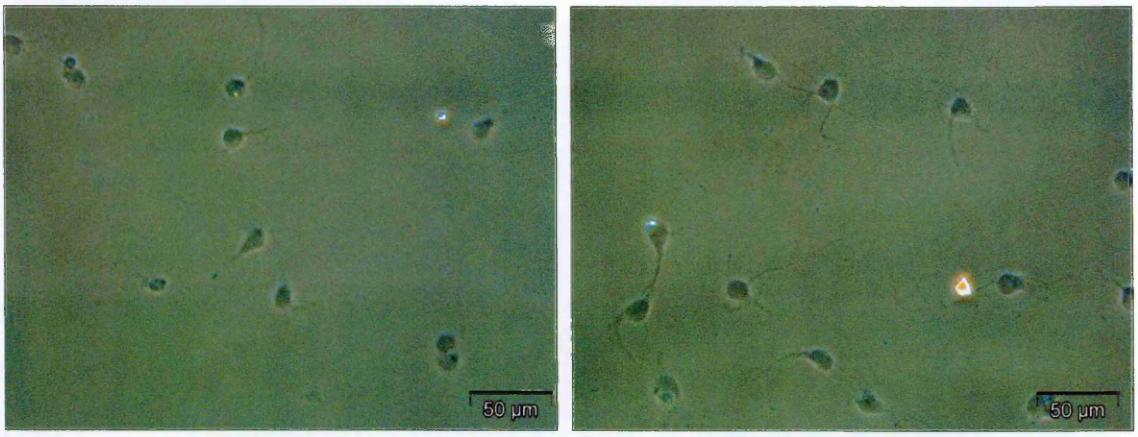


Figure 4-11: Quantification of the number of GFAP positive cells present in primary E18 rat hippocampal cultures at 20,000 cells/well under treatment conditions with 100ng/ml CLN, 10µM menadione or both together. 100ng/ml CLN alone had no effect on the number of GFAP positive astrocytes in these cultures. In cultures treated with CLN and menadione the number of GFAP positive astrocytes was slightly reduced compared to control cultures and menadione treated cultures. n=3 with at least 3 replicates per condition per experiment. Errors bars represent standard errors.



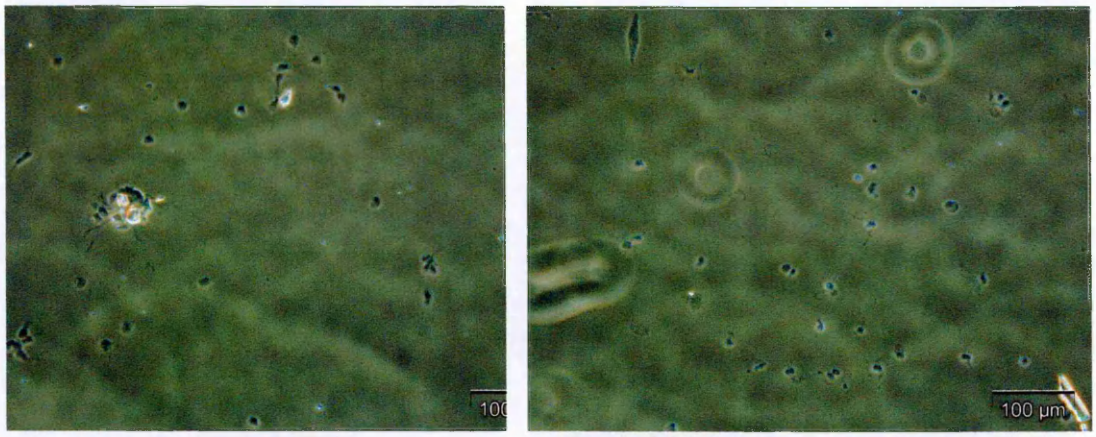
A: Control B: 100ng/ml CLN

Figure 4-12: Phase contrast images of control (A) cultures and cultures treated with 100ng/ml CLN (B) from E18 hippocampi, plated at 20,000 cells/well and fixed at *DIV*4. This shows that CLN had no notable effect on neuritres.

Higher concentrations of CLN (1µg/ml) alone also showed no effect on the morphology of MAP-2 or GFAP positive cells compared to control cultures (see Figures 4-8A, 4-9A, 4-14A and 4-15A). Quantification confirmed that treatment with 1µg/ml CLN did not affect the number of MAP-2 positive neurons (see Figure 4-16) or GFAP positive astrocytes (see Figure 4-17).

This higher concentration of bovine CLN offered a small effect on the menadione-induced reduction in MAP-2 positive neurons but this was not significant. Menadione casued a 60±30% decrease in MAP-2 positive neurons and the increase in MAP-2 positive neuron numbers when 1µg/ml CLN was given with menadione compared to menadione treatment alone was not significant (P = 0.517.see Figures 4-16).

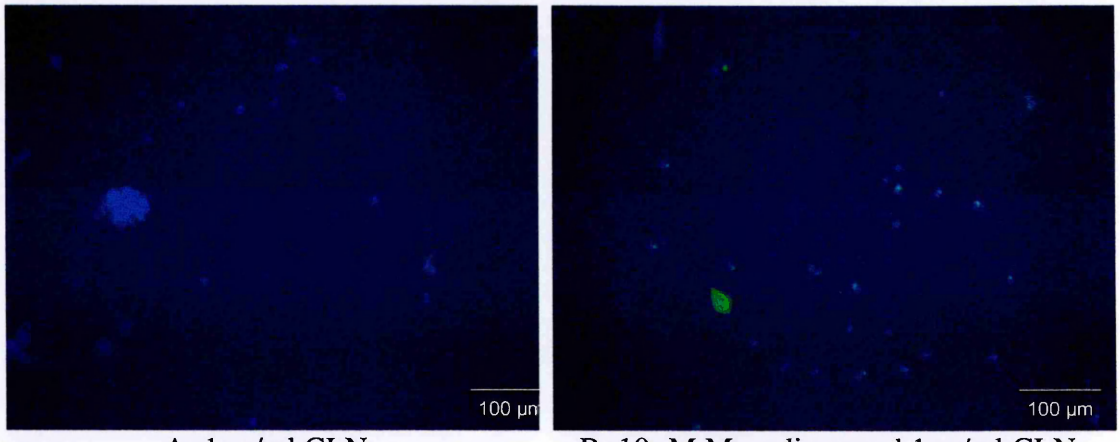
Phase contrast: cultures at 20,000 cells/well



A: 1µg/ml CLN B:10µM Menadione and 1µg/ml CLN

Figure 4-13: Phase contrast images 20,000 cells/well cultures from E18 hippocampi treated with 1µg/ml CLN (A) or 10µM menadione with 1µg/ml CLN (B) for 24 hours. The total number of cells present in the culture was not different compared to control (Figure 4-7A) in cultures treated with 1µg/ml CLN (A).

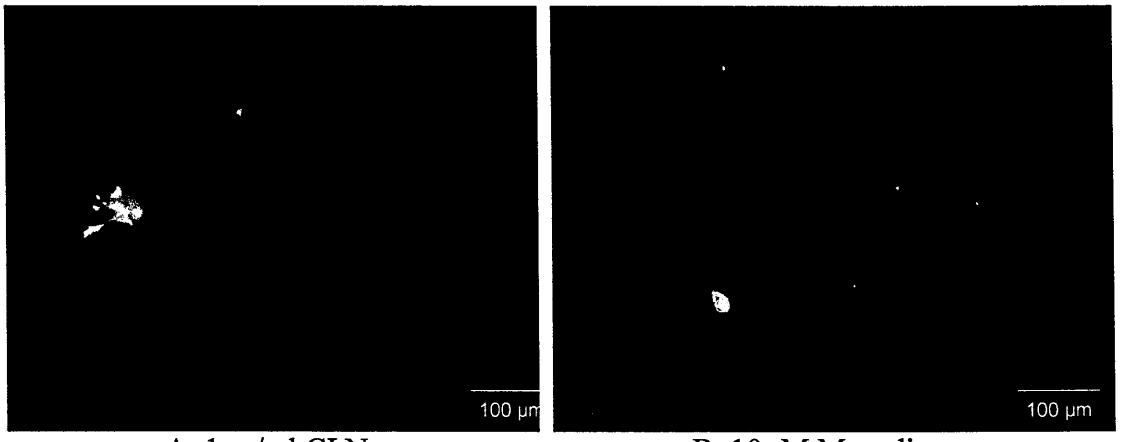
MAP-2 positive neurons: cultures at 20,000 cells/well



A: 1µg/ml CLN B: 10µM Menadione and 1µg/ml CLN

Figure 4-14: MAP-2 immunolabelling images of 20,000 cells/well cultures from E18 hippocampi treated with 1µg/ml CLN (A) or 10µM menadione with 1µg/ml CLN (B) for 24 hours. The morphology and number of MAP-2 positive neurons in cultures treated with 1µg/ml CLN (A) did not differ from control cultures (Figure 4-8A). The menadione-induced reduction in the number of MAP-2 positive cells (Figure 4-8C) was only slightly affected when 1µg/ml CLN was given with menadione (B).

GFAP positive astrocytes: cultures at 20,000 cells/well



A: 1µg/ml CLN

B: 10µM Menadione and 1µg/ml CLN

Figure 4-15: GFAP immunolabelling images of 20,000 cells/well cultures from E18 hippocampi treated with 1µg/ml CLN (A) or 10µM menadione with 1µg/ml CLN (B) for 24 hours. Treatment with 1µg/ml CLN (A) had no effect on GFAP positive astrocytes. Upon treatment with 10µM menadione and 1µg/ml CLN (B) GFAP positive astrocytes were unaffected compared to control cultures (Figure 4-9A) and slightly increased compared to menadione treated cultures (Figure 4-9C).

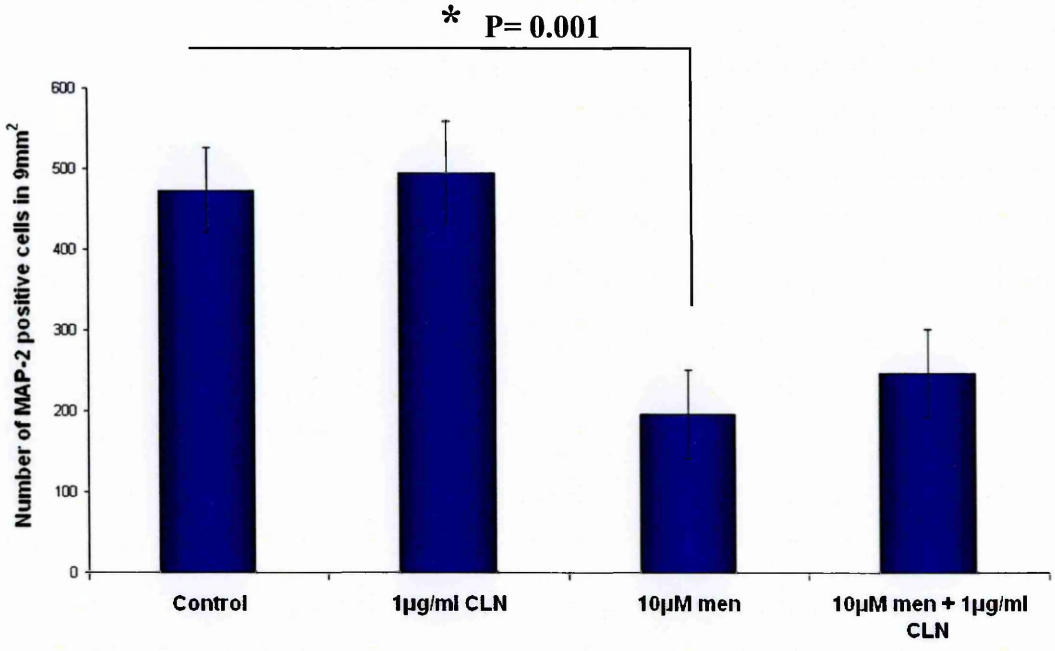


Figure 4-16: Quantification of the number of MAP-2 positive cells present in primary E18 rat hippocampal cultures at 20,000 cells/well treated with 1µg/ml CLN, 10µM menadione or both together. CLN alone had a no effect on the number of MAP-2 positive neurons in these cultures. Menadione treatment caused a 90±22% reduction in the number of MAP-2 positive neurons present in the cultures (from one-way ANOVA P = 0.001) and CLN partially protected against this reduction (from one-way ANOVA P = 0.517). n=3 with at least 3 replicates per condition per experiment. Errors bars represent standard errors. * represents P < 0.01.

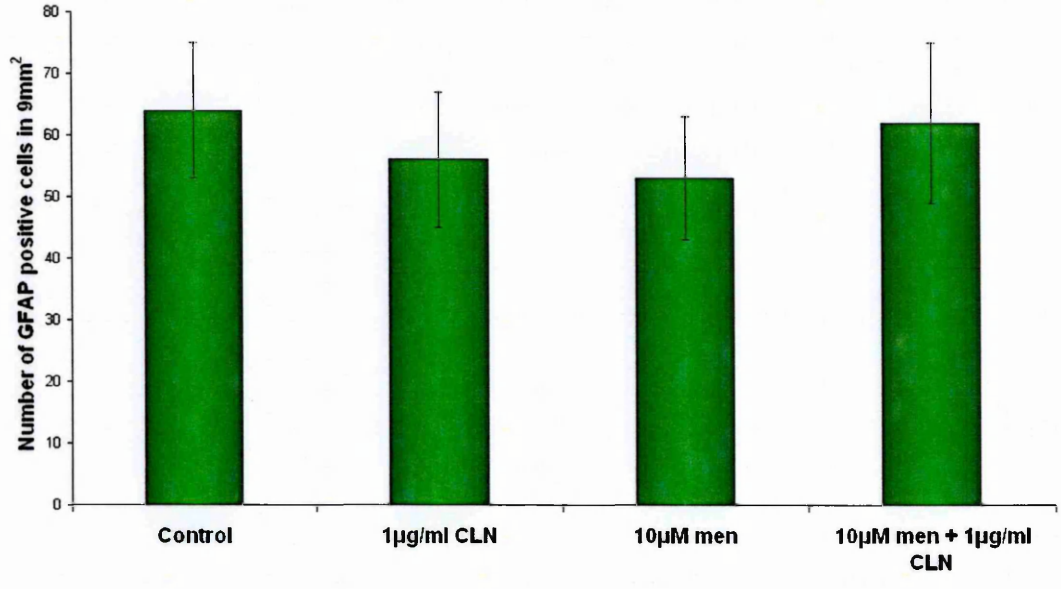


Figure 4-17: Quantification of the number of GFAP positive cells present in primary E18 rat hippocampal cultures at 20,000 cells/well treated with 1µg/ml CLN, 10µM menadione or both together. Neither 1µg/ml CLN nor 10µM menadione had any notable effect on the number of GFAP positive astrocytes present in these cultures. n=3 with at least 3 replicates per condition per experiment. Errors bars represent standard errors.

4.4.1.2. The effect of CLN on menadione-induced cytotoxicity in the B50 cell line

In order to assess the effects of bovine CLN alone and against menadione-induced toxicity in the B50 cell line an MTS cytotoxicity assay was carried using five different concentrations of CLN ranging between 10ng/ml and 10μg/ml. These CLN treatments were given alone or with menadione at 10 or 20μM.

The results showed that bovine CLN at concentrations ranging between 10ng/ml and 10μg/ml has no trophic effect in terms of cell number on B50 cells in culture (see Figures 4-18 to 4-22).

Menadione for 24 hours consistently induced toxicity, by 50-60% upon treatment with 10μM menadione and 71-75% upon treatment with 20 μM menadione when compared to controls. Bovine CLN did not prevent this menadione-induced cell loss at any of the concentrations of CLN used whether given prior to or along with menadione treatment (see Figures 4-18 to 4-22) although CLN at 10ng/ml had a very small effect against toxicity induced by 20μM menadione treatment (see Figure 4-18).

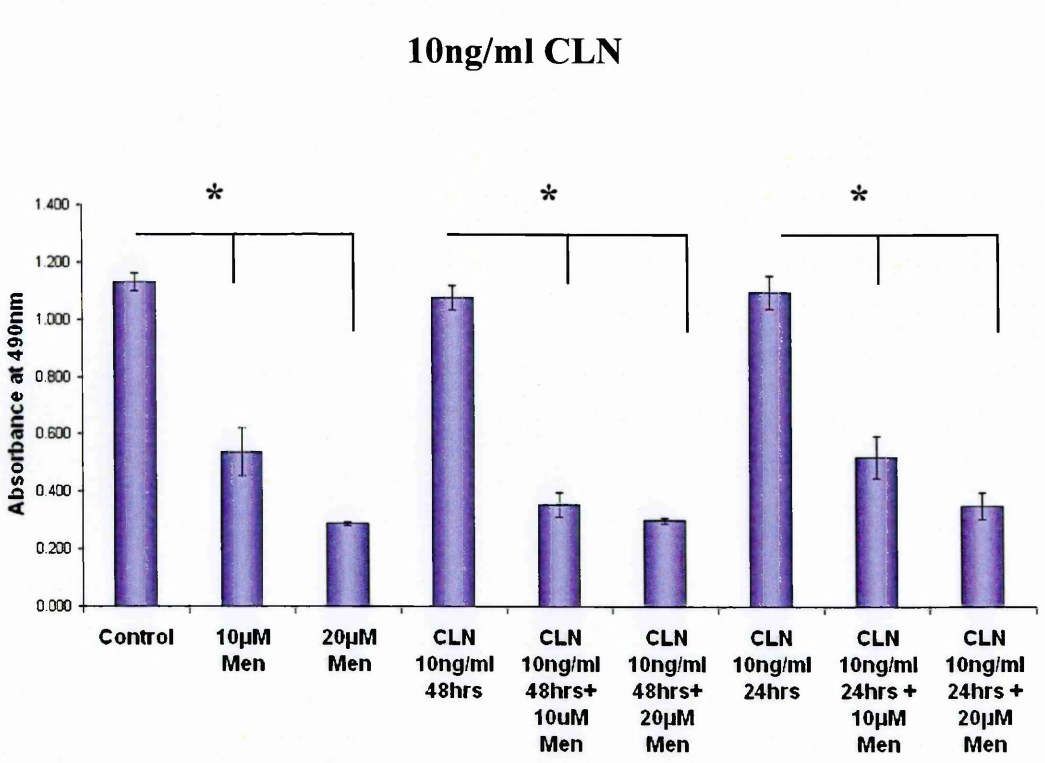


Figure 4-18: MTS assay absorbance values at 490nm on B50 cells treated with menadione and 10ng/ml bovine CLN. This shows that 10 or 20µM menadione caused a reduction in absorbance at 490nm compared to control and CLN treated cultures (from one-way ANOVA $P < 0.001$). 10ng/ml CLN did not protect against menadione-induced cytotoxicity despite a very small improvement when CLN was given with menadione compared to 20µM menadione alone (from one-way ANOVA $P > 0.9$). * is represents $P < 0.001$.

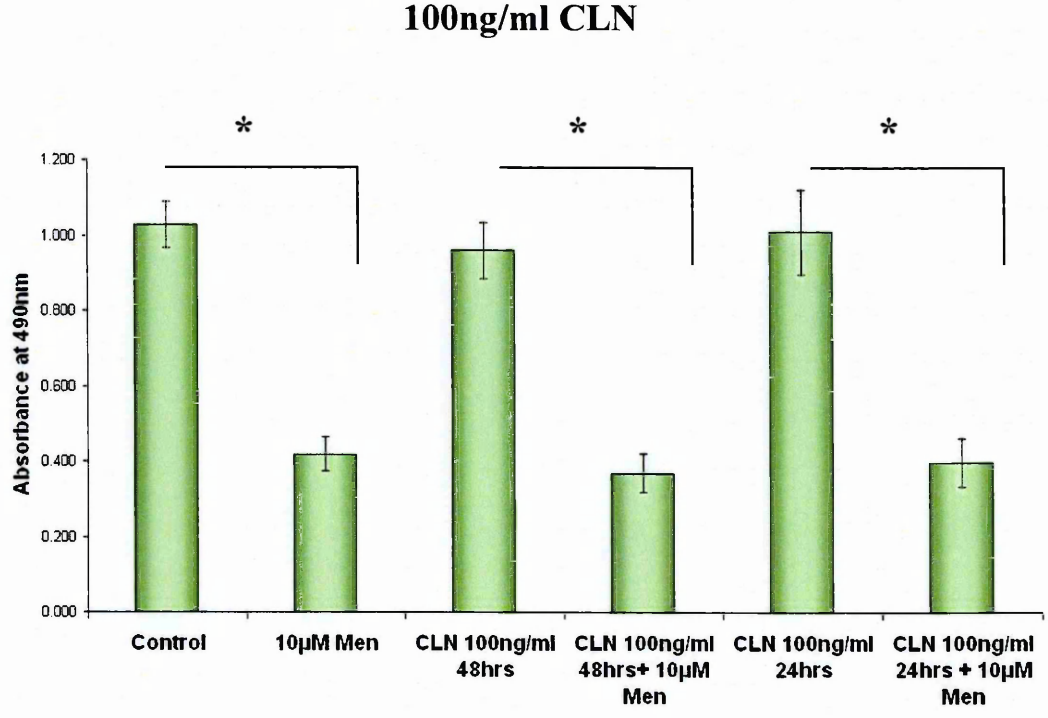


Figure 4-19: MTS assay absorbance values at 490nm on B50 cells treated with menadione and 100ng/ml bovine CLN. This shows that 10µM menadione caused a reduction in absorbance at 490nm compared to control and CLN treated cultures (from one-way ANOVA $P < 0.001$). 100ng/ml CLN did not protect against menadione-induced cytotoxicity (from one-way ANOVA $P > 0.9$). * represents $P < 0.001$.

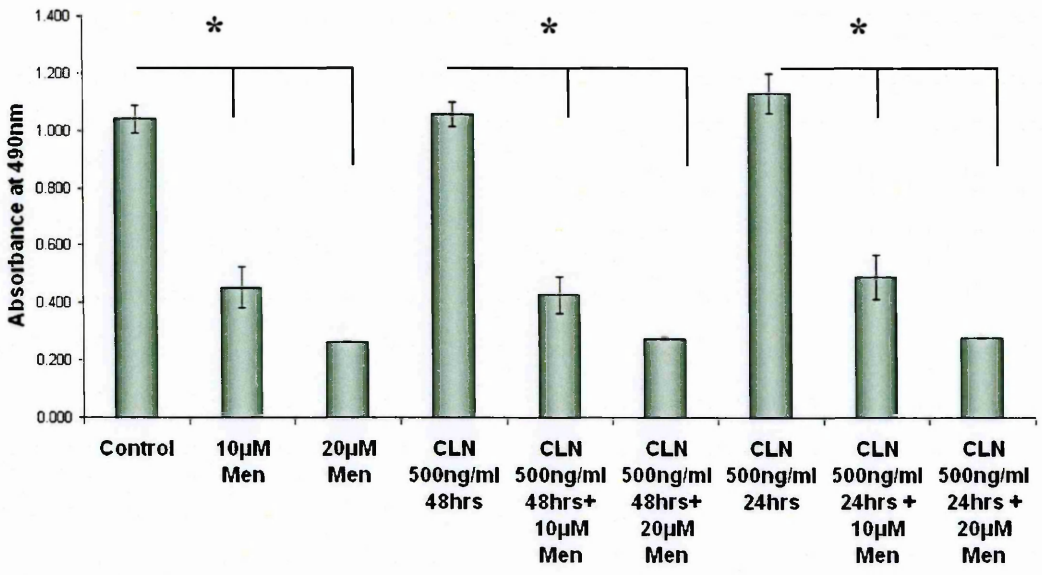


Figure 4-20: MTS assay absorbance values at 490nm on B50 cells treated with menadione and 500ng/ml bovine CLN. This shows that 10µM menadione caused a reduction in absorbance at 490nm compared to control and CLN treated cultures (from one-way ANOVA $P < 0.001$). 100ng/ml CLN did not protect against menadione-induced cytotoxicity (from one-way ANOVA $P > 0.9$). * represents $P < 0.001$.

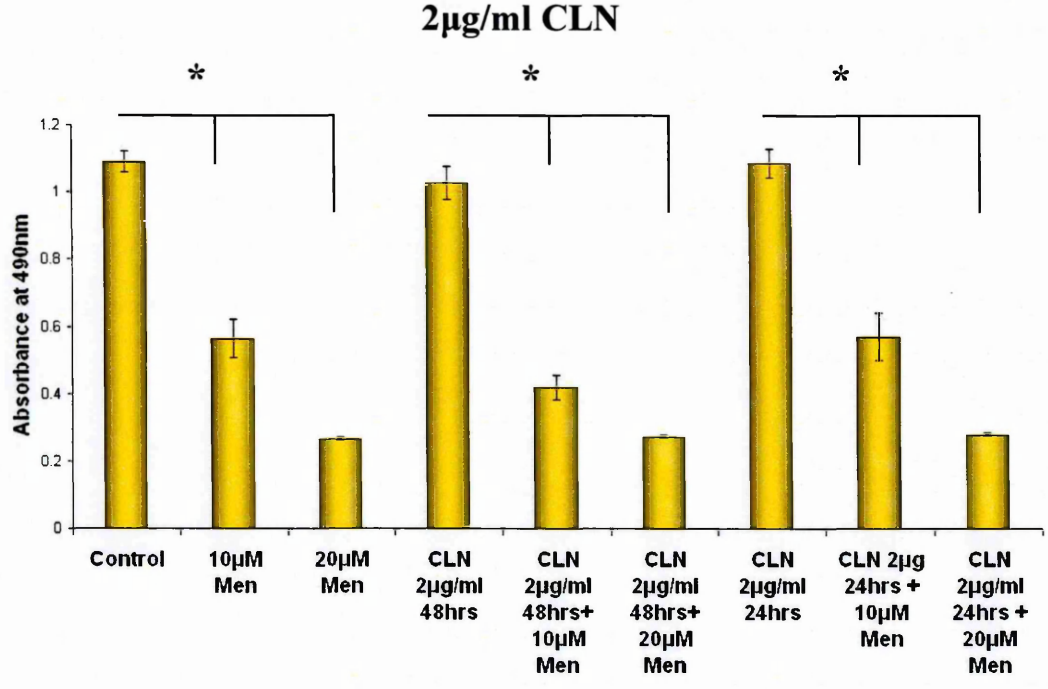


Figure 4-21: MTS assay absorbance values at 490nm on B50 cells treated with menadione and 2µg/ml bovine CLN. This shows that 10 and 20µM menadione caused a reduction in absorbance at 490nm compared to control and CLN treated cultures (from one-way ANOVA $P < 0.001$). 2µg/ml CLN did not protect against menadione-induced cytotoxicity (from one-way ANOVA $P > 0.9$). * represents $P < 0.001$.

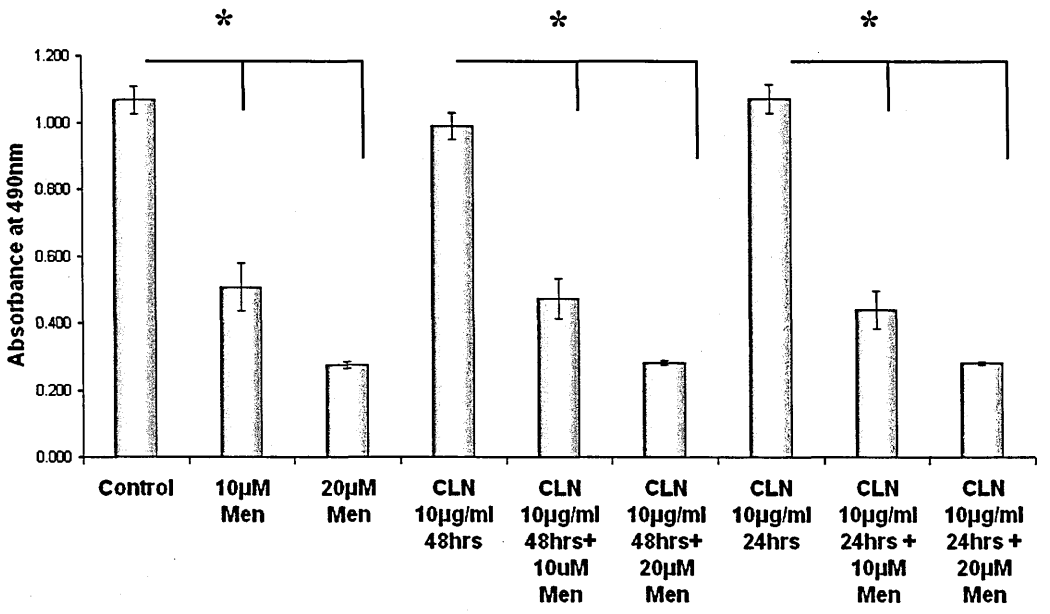


Figure 4-22: MTS assay absorbance values at 490nm on B50 cells treated with menadione and 10µg/ml bovine CLN. This shows that 10 and 20µM menadione caused a reduction in absorbance at 490nm compared to control and CLN treated cultures (from one-way ANOVA $P < 0.001$). 10µg/ml CLN did not protect against menadione-induced cytotoxicity (from one-way ANOVA $P > 0.9$). * represents $P < 0.001$.

4.4.2. The effect of CLN against beta-amyloid-induced toxicity

4.4.2.1. Changes in neuronal morphology upon treatment with bovine CLN and/or beta-amyloid$_{1-42}$

On visual inspection of the cultures, 48 hour 25µM Aβ_{1-42} treatment had a marked effect on the morphology of the MAP-2 positive neurons that were present (see Figure 4-24A and C). Neurons in control cultures treated with PBS and in cultures treated with 5µg/ml bovine CLN for 48 hours had rounded morphology and extended several processes (see Figure 4-24A and B). However neurons in cultures treated with Aβ_{1-42} lacked processes and appeared shrunken (see Figures 4-23C and 4-24C). These changes in morphology were still present when 5µg/ml bovine CLN was pre-incubated and co-administered with Aβ_{1-42} (see Figure 4-24D).

The morphology of GFAP positive astrocytes was not altered upon treatment with $A\beta_{1-42}$ compared to controls (see Figure 4-25A and C). $A\beta_{1-42}$ caused a reduction in the total number of cells present in cultures compared to controls (see Figure 4-23A and C).

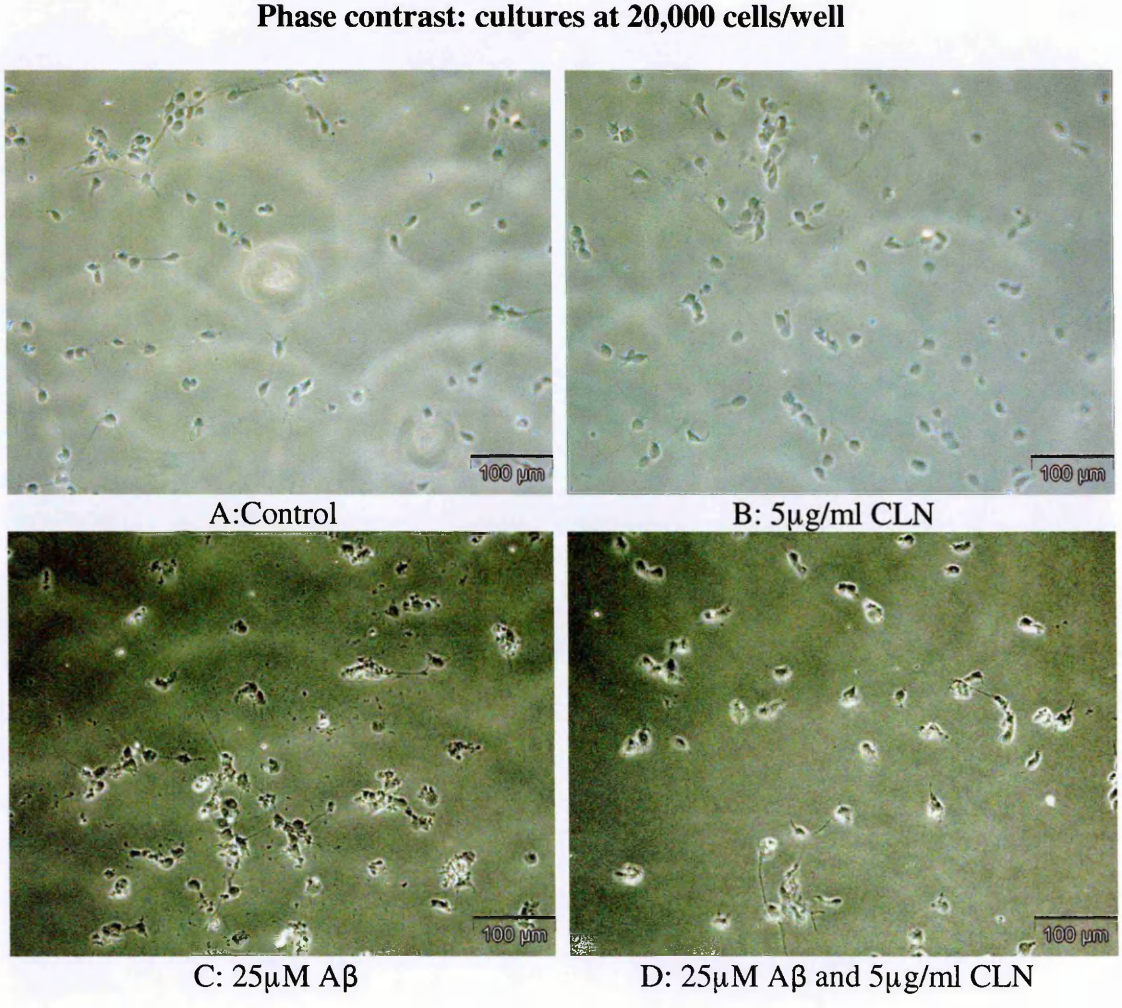


Figure 4-23: Phase contrast images of 20,000 cells/well cultures from E18 hippocampi treated with PBS (A), 5µg/ml CLN (B), 25µM $A\beta_{1-42}$ (C) or 5µg/ml CLN and 25µM $A\beta_{1-42}$ (D) for 48 hours. Control cultures had rounded cells with processes as well as a few flat cells (A). Treament with 5µg/ml CLN (B) didn't affect the total number of cells present in the cultures compared to controls. However, treatment with $A\beta_{1-42}$ (C) caused a reduction in the total number of cells present in the culture compared to control (A). Pre-incubation and co-administration of CLN with $A\beta_{1-42}$ only had a small effect against the $A\beta_{1-42}$-induced reduction in the number of cells (D).

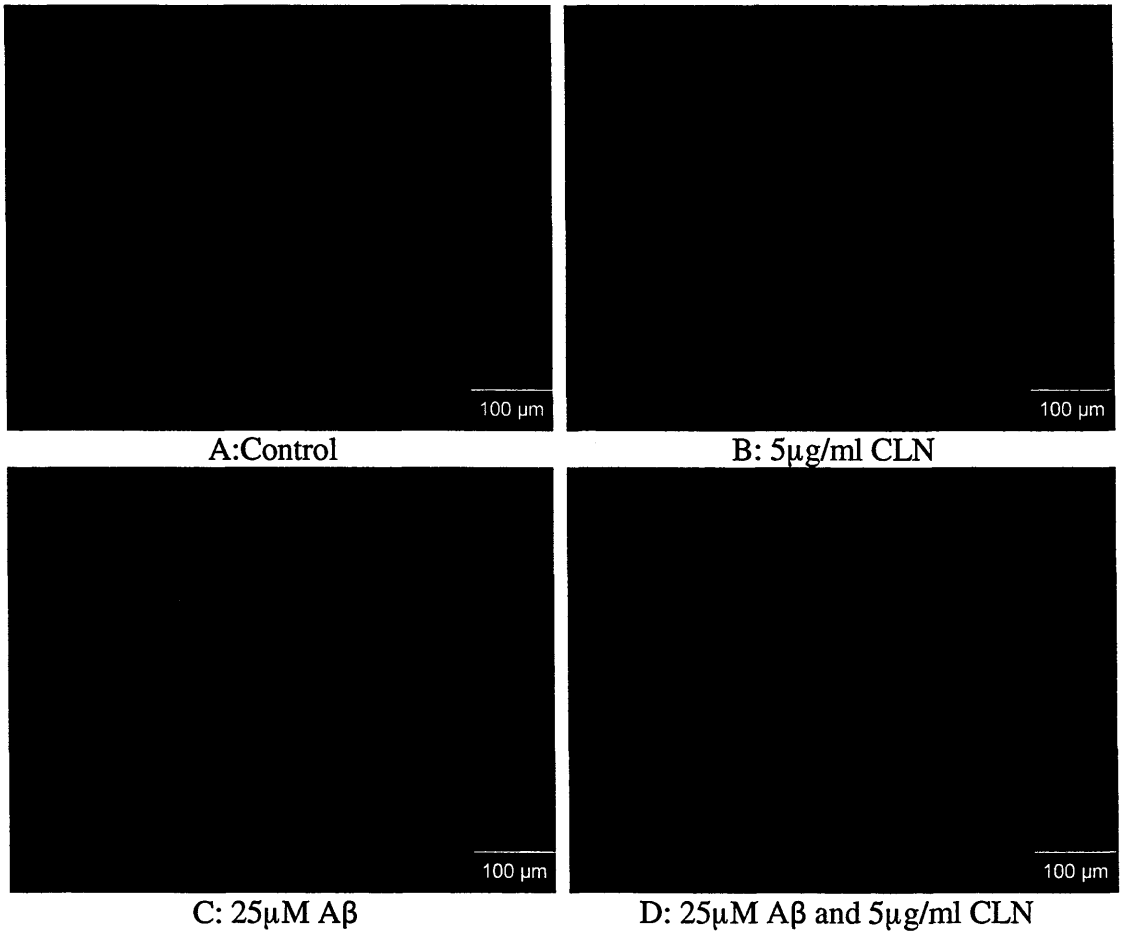


Figure 4-24: MAP-2 immunolabelling images of 20,000 cells/well cultures from E18 hippocampi treated with PBS (A), 5µg/ml CLN (B), 25µM $A\beta_{1-42}$ (C) or 5µg/ml CLN and 25µM $A\beta_{1-42}$ (D) for 48 hours. The morphology and number of MAP-2 positive neurons in cultures treated with 5µg/ml CLN (B) did not differ from control cultures. However, treatment with $A\beta_{1-42}$ (C) led to a change in morphology compared to controls (A). Pre-incubation and co-administration of CLN with $A\beta_{1-42}$ only had little effect against the $A\beta_{1-42}$-induced change in cell morphology (D).

GFAP positive astrocytes: cultures at 20,000 cells/well

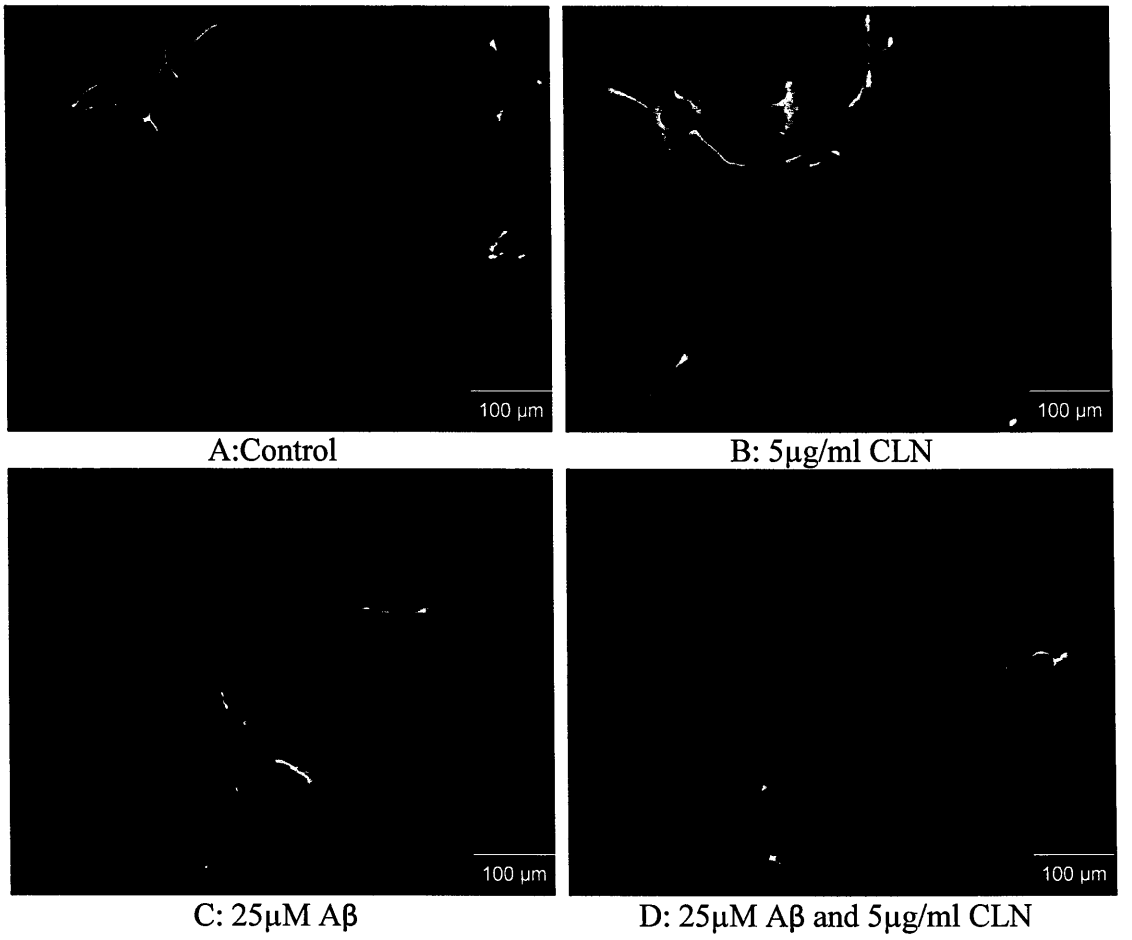


Figure 4-25: GFAP immunolabelling images of 20,000 cells/well cultures from E18 hippocampi treated with PBS (A), 5µg/ml CLN (B), 25µM $A\beta_{1-42}$ (C) or 5µg/ml CLN and 25µM $A\beta_{1-42}$ (D) for 48 hours. The morphology GFAP positive astrocytes in cultures treated with 5µg/ml CLN (B) or 5µg/ml CLN and 25µM $A\beta_{1-42}$ (C) did not differ from control cultures.

4.4.2.2. Changes in cell survival in culture upon treatment with beta-amyloid with or without CLN

Quantification revealed a significant level of cell loss upon treatment of dissociated hippocampal cultures with Aβ $_{1-42}$. Treatment with 5μg/ml CLN alone had no significant effect on the number of MAP-2 cells present in the cultures but treatment with 25μM Aβ$_{1-42}$ for 48 hours resulted in a significant 44 ±13% (SE) (P = 0.003) reduction of MAP-2 positive cells compared to control cultures (see Figure 4-26). A small non-significant increase in MAP-2 positive cells when CLN at 5μg/ml was pre-incubated and administered along with Aβ$_{1-42}$ compared to Aβ$_{1-42}$ alone was also observed (see Figure 4-26).

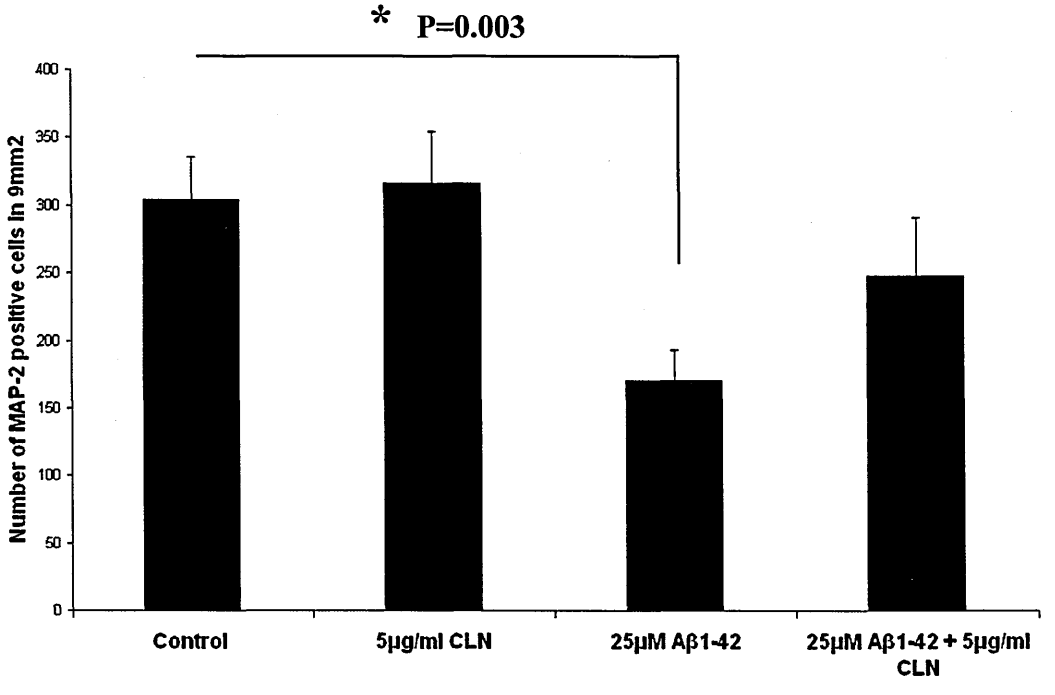


Figure 4-26: Quantification of the number of MAP-2 positive neurons in dissociated hippocampal cultures at 20,000 cells/well treated with Aβ $_{1-42}$ and CLN. The number of MAP-2 positive cells was relatively unchanged after 48 hours treatment with 5μg/ml CLN alone compared to PBS treated controls (from one-way ANOVA P= 0.804). However the number of neurons decreased when cultures were treated with 25μM Aβ $_{1-42}$ alone (from one-way ANOVA P = 0.03). n=4 with at least 3 replicates per condition in each experiment. Error bars represent standard errors. * indicates P= 0.003.

Treatment of cultures with 25μM Aβ $_{1-42}$ for 48 hours also led to a reduction in the number of GFAP positive cells present in the cultures (see Figure 4-27). This decrease in the number of GFAP positive cells was less when CLN at 5μg/ml was given along with 25μM Aβ$_{1-42}$ compared to 25μM Aβ $_{1-42}$ alone (see Figure 4-27).

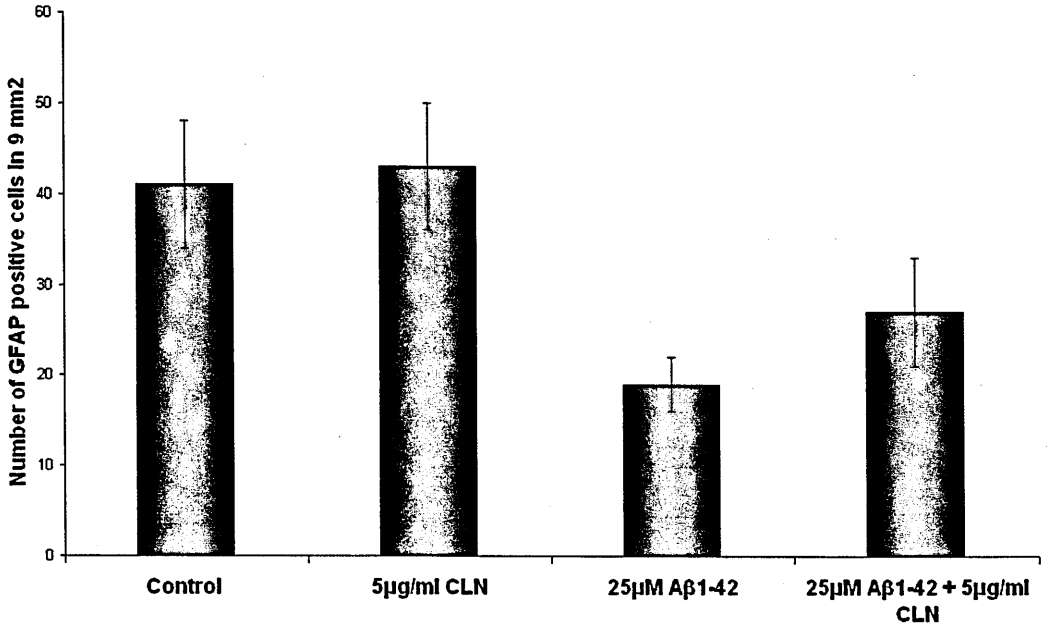


Figure 4-27: Quantification of the number of GFAP positive astrocytes in dissociated hippocampal cultures at 20,000 cells/well treated with Aβ$_{1-42}$ and CLN. The number of GFAP positive cells was relatively unchanged after 48 hours treatment with 5μg/ml CLN alone compared to PBS treated controls. However the number of astrocytes decreased when cultures are treated with 25μM Aβ $_{1-42}$ alone (from one-way ANOVA P = 0.088) and CLN gave only a minimal protective effect against this Aβ$_{1-42}$ induced toxicity. n=4 with at least 3 replicates per condition in each experiment. Error bars represent standard errors.

4.5. Discussion

4.5.1. The effect of CLN against menadione-induced toxicity in primary hippcoampal cells

The first experiments described in this chapter were designed to study the effect of the oxidative stress inducer, menadione, on primary hippocampal cells and the B50 cell line in culture. This oxidative stress-mediated model of cell death allowed investigation into the potential of bovine CLN to alleviate oxidative stress-induced cell death.

The results showed that, in low density cultures treated with 100ng/ml bovine CLN alone there was a 27±13% increase in MAP-2 positive neurons in these cultures compared to controls. This small effect may be due to CLN causing the release of trophic factors into the culture media. This hypothesis receives support from previous work showing that CLN can upregulate the expression of the platelet derived growth factor (PDGF) in TR146 mucosal cells (Szaniszlo *et al.* 2009). However, in the work described here, 1µg/ml CLN had no trophic effect on cell numbers and from the results presented in this Chapter, Section 4.1.1.1 and in Chapter 3, CLN at 100ng/ml did not have any effect on cell morphology or numbers in cultures from E18 hippocampi plated at higher densities. The specificity of the conditions needed in order to see the small effect on cell numbers might be due to 100ng/ml being an optimal concentration of CLN for causing a trophic effect and improved background cell survival in higher density cultures compared to those seeded at low density because of cell-cell interaction. Improved background cell survival would make a trophic effect less apparent. Alternatively the effect may be due to the release of a trophic factor and this may then be less concentrated on the individual cells in higher density cultures compared to low density cultures.

As expected from previous results, menadione at 10μM caused a significant reduction in the number of MAP-2 positive neurons present in primary hippocampal cultures. This cell loss was particularly severe in the lower density, (20,000 cells/well), cultures. The cell loss is likely to be mainly due to cell death by necrosis as it has also been found by Adamec *et al.* (2000) that a 10μM concentration of menadione, which is considered a high dose, cell death is largely necrotic rather than apoptotic (these forms of cell death and their differences were discussed in detail in Chapter 1, Section 1.3.1 and 1.3.2) which is the case with lower doses of menadione (1μM) (Adamec *et al.* 2000). However, it has also been found in previous studies that over shorter time periods of up to 60 minutes much higher doses of menadione, in the range of 40-100μM, have led to the increased expression of the apoptotic marker annexinV (Chiou *et al.* 2003). This implies that some apoptosis is likely to be occurring at the mid-range concentration (10μM) of menadione used in these experiments. 10μM menadione was used for the cell death model described in this Chapter because it has been found to cause more marked cell death in a shorter time period than lower concentrations of menadione (Adamec *et al.* 2000).

Although there was a small reduction in the number of GFAP positive astrocytes in low density cultures in response to menadione treatment, this was not as marked as the reduction in MAP-2 positive neurons that was observed and only led to a small, non-significant reduction in the number of GFAP positive astrocytes present in these cultures. This result implies that the toxicity of menadione is neuron-specific. Although this lack of glial cell loss upon treatment with menadione could potentially be a glial scar-like effect, whereby cell stress leads to proliferation of astrocytes (Faijerson *et al.* 2006), this would lead to an increase in the number of GFAP positive cells rather than no change. It is therefore most likely due to increased resistance of astrocytes to oxidative stress induced damage. This latter

hypothesis is in agreement with previous work by Hollensworth *et al.* (2000) showing that GFAP positive astrocytes were less susceptible to menadione-induced injury and had more efficient repair systems than oligodendrocytes or microglia (Hollensworth *et al.* 2000).

This work examining the effects of bovine CLN against menadione-induced oxidative stress on primary hippocampal cells has shown that at the concentrations used (100ng/ml and 1μg/ml) CLN has only a small effect which is non-significant on the oxidative stress-related damage caused by treatment with menadione. The protective effect was most apparent in low-density (20,000 cells/well) cultures where 100ng/ml CLN given along with 10μM menadione increased the number of MAP-2 positive cells to 20±3% of controls compared with 10±0.2% in cultures treated with menadione alone.

4.5.2. The effect of CLN against menadione-induced toxicity in the B50 cell line

The B50 cell line has previously been used for oxidative stress experiments using H_2O_2 and it was found that a dose of 100μM for 24 hours caused a 40-50% decrease in cell viability (Iwata *et al.* 1998; Miyazaki *et al.* 1999). These cells have therefore been shown to be susceptible to the damage by oxidative stress in culture and were a good choice for further study of the possible protective effects of CLN as described in Section 4.1. The results showed a 50-60% decrease in cell viability with 10μM menadione and 71-75% decrease in viability with 20μM menadione over 24 hours. CLN treatment along with menadione was not able to prevent the menadione-induced cell death at any of the concentrations used.

The results from the experiments on the B50 cell line demonstrated that at the concentrations tested (10ng/ml-10μg/ml) bovine CLN alone is not trophic to these cells in culture. This observation appears to be contrary to the study by Bacsi *et al.*

(2005) showing CLN to have some trophic effects and increase neurite length in the PC12 cell line by activating P_{53} and P_{21}. However, the study by Bacsi *et al*, (2005) involved study of cell culture over considerably longer time scales than the work described here. Furthermore, different cell lines may react differently to treatment even though they are both neuronal lines derived from rat tissue, because the PC12 cell line is derived from rat adrenal medulla and the B50 cell line is derived from rat hippocampus.

4.5.3. The effect of CLN against $A\beta_{1-42}$-induced toxicity in primary hippocampal cells

The experiments described here are the first to examine the effects of CLN on primary hippocampal cells that have been treated with Aβ. As CLN has been shown to prevent the toxicity of $A\beta_{1-40}$ on SHSY-5Y cells in culture when pre-incubated and co-administered with $A\beta_{1-40}$ (Schuster *et al.* 2005) it was considered important to know whether this protection by CLN also occurs in primary cell cultures treated with Aβ. Therefore this experiment was designed to replicate this previous work on cell lines using primary hippocampal cells. As described in Section 4.2.4 $A\beta_{1-42}$ was chosen because of its preparation procedure and faster induction of toxicity over $A\beta_{1-40}$. $A\beta_{1-42}$ was pre-incubated with or without bovine CLN for 48 hours prior to cell treatment. The cells were then treated for 48 hours before being fixed and stained for the neuronal marker MAP-2 and the astrocytic marker GFAP.

CLN was prepared slightly differently for the experiments with $A\beta_{1-42}$ compared to those with menadione (see Section 4.3.1) and was used at a higher concentration (5μg/ml compared to 100ng/ml and 1μg/ml). The results presented

here show that CLN diluted in PBS and media at 5μg/ml and given alone had no trophic effect on these cells.

Forty eight hour treatment with 25μM $A\beta_{1-42}$ caused a significant 44±13% decrease in the number of MAP-2 positive neurons present in these primary cell cultures compared to controls. This is in keeping with many previous studies showing the toxicity of $A\beta_{1-42}$ on primary hippocampal cells in culture as discussed in Section 4.2.3.

When bovine CLN was pre-incubated with $A\beta_{1-42}$ there was a non-significant trend against the $A\beta_{1-42}$-induced decrease in MAP-2 positive neurons, implying that CLN at this concentration may have a small effect against $A\beta_{1-42}$-induced neurotoxicity. The effect seen here is not as dramatic as the significant protective effect of CLN seen previously by Schuster *et al* (2005) and there may be several reasons for this difference; firstly, the effect that was shown by Schuster *et al.* used a cell line and here the effect of CLN on primary hippocampal cells was being investigated for the first time. Furthermore the authors in the preceding work had used $A\beta_{1-40}$ treatment whereas $A\beta_{1-42}$ was used here. As explained in the introduction $A\beta_{1-42}$ was chosen for these experiments because $A\beta_{1-42}$ has been shown to be more toxic to primary hippocampal cells in culture than $A\beta_{1-40}$ and $A\beta_{1-42}$ can be dissolved in water and PBS rather than acid.

Bovine CLN alone was seen to have no significant effect on the number of GFAP positive cells present in the hippocampal cultures although there was small but non-significant increase in cell numbers. This result supports data in Chapter 3, Section 3.6 showing that lower concentrations of bovine CLN had no notable effect on the number of GFAP positive astrocytes. However $A\beta_{1-42}$ treatment led to a 50% reduction in the number of GFAP positive cells present in cultures. This is in keeping with work done by Assis-Nascimento *et al.* in 2007 showing that embryonic rat

astrocytes in culture are vulnerable to the toxicity of Aβ (Assis-Nascimento *et al.* 2007). This $A\beta_{1-42}$ induced reduction in GFAP positive cell numbers was not reversed by the presence of CLN pre-incubated with $A\beta_{1-42}$ showing that CLN was not able to protect astrocytes from the effects of $A\beta_{1-42}$ at these concentrations and in these treatment conditions. It is entirely possible though that although CLN cannot protect the astrocytes, *per se*, the small effect of CLN seen on neurons may occur via the astrocytes. Evidence in support of this theory comes from work showing that other protective compounds such as LPS and IFN-γ have been found to help prevent Aβ-mediated neurotoxicity via changes in the activity of astrocytes and the release of protective factors (Ramirez *et al.* 2005). Therefore the small increase in the number of GFAP positive astrocytes with CLN alone may be interesting regarding the prevention of neurotoxicity because an increase in the number of astrocytes might have an indirect effect on neuronal survival. However, there is work by Domenici *et al.* (2002), which would argue against this hypothesis as these authors showed that the presence of astrocytes in cultures may actually increase neuronal vulnerability to Aβ-induced neurotoxicity, and pure neuronal cultures where astrocytic growth was prevented by treatment with arabinosylcytosine were far less vulnerable than mixed cultures (Domenici *et al.* 2002).

4.5.4. Summary

These experiments have demonstrated that bovine CLN may have a small effect against menadione-induced toxicity and $A\beta_{1-42}$-induced toxicity on MAP-2 positive neurons in primary hippocampal cultures but has less of a protective effect against the $A\beta_{1-42}$-induced toxicity on GFAP positive astrocytes. Bovine CLN did not protect B50 cell cultures from menadione-induced toxicity.

Chapter 5

Investigation of the mechanism of action of CLN

5.1. Aims

The aim of these experiments was to elucidate the mechanism of the protective effects of CLN that have been observed against both menadione and Aβ-induced toxicity on neuronal cells. There were three aspects involved in this analysis and these are illustrated in Figure 5-1.

Firstly, the effect of CLN on oxidative stress and antioxidant defence was investigated by analysing the ability of CLN to prevent the production of ROS and to affect the protein levels of the antioxidant enzyme SOD1.

Secondly, the effect of bovine CLN on the protein kinase Cdk5 was studied. Cdk5 has been demonstrated to be involved in Aβ-induced toxicity and to be inhibited by TNFα.

Thirdly, the effect of bovine CLN on apoptosis was investigated by FACS analysis of the levels of activated caspase 3 in cultures upon treatment with bovine CLN and 1μM menadione.

The primary hippocampal cultures described in Chapter 3 and B50 cells were used as the model systems.

5.2. Introduction

5.2.1. Oxidative stress markers *in vitro*

An increase in the levels of activity or protein expression of anti-oxidant enzymes such as SOD in cultures would indicate that the cells are responding to some form of oxidative stress. Measurement of these parameters can be carried out using Western blotting to investigate changes in protein levels or assays to examine changes in enzyme activity. Furthermore changes in the levels of ROS being produced may be directly measured using DCFH-DA (Hempel *et al.* 1999; Halliwell and Whiteman 2004). DCFH-DA can freely enter cells where it reacts with esterases to produce an insoluble form of the molecule which can then react with peroxides to leave a fluorescent compound DCF, the intensity of which may be measured using a confocal microscope (Jakubowski and Bartosz 2000).

5.2.2. Protection against beta-amyloid-induced toxicity

There are several aspects of the mechanisms of Aβ-induced toxicity upon which a compound may act in order to prevent the toxicity. This effect may be directly on Aβ and its production or aggregation or it may be more indirect.

The importance of the aggregation state on the level of toxicity induced has been emphasised both in Chapter 1, Section 1.1.2.2 and in Chapter 4, Section 4.2.3. As detailed in Chapter 1, Section 1.1.5.1 compounds such as trehalose (Liu *et al.* 2005), NAP (Ashur-Fabian *et al.* 2003) and the polyphenol group (Riviere *et al.* 2007) are protective against Aβ-induced toxicity by preventing this aggregation.

The protective effects of a compound may also act upon one of the pathways which has been implicated in Aβ-induced toxicity as was discussed in detail in Chapter 1, Section 1.1.5.2. Furthermore anti-oxidants have been shown to be

protective against Aβ-induced toxicity as detailed in Chapter 1, Section 1.3.4 and compounds may protect against Aβ-induced toxicity by preventing this Aβ-mediated induction of apoptosis as discussed in detail in Chapter 1, Section 1.2.4.

Some compounds may be protective against Aβ-induced toxicity by combinations of these effects. Components of green and black teas have been found to be protective against Aβ-induced toxicity in hippocampal cultures by decreasing ROS accumulation as well as preventing apoptosis and $A\beta_{25-35}$ fibril formation (Bastianetto *et al.* 2006) and the red wine associated antioxidant, resveratrol, has been shown to prevent both ROS accumulation and apoptosis initiation by $A\beta_{25-35}$ in PC12 cells (Jang and Surh 2003).

5.2.3. Mechanisms of protection by CLN

There are numerous potential mechanisms by which CLN may mediate its protective effects against menadione and $A\beta_{1-42}$-induced toxicity and some of these are outlined in Figure 5-1.

As discussed in Chapter 1, Section 1.5.4 CLN has been shown to prevent the toxicity of $A\beta_{1-40}$ in SHSY-5Y cells by inhibiting and even reversing the aggregation of $A\beta_{1-40}$ (Schuster *et al.* 2005). This ability of CLN to prevent $A\beta_{1-40}$ fibril formation was confirmed by (Bourhim *et al.* 2007) and this known action of CLN is shown in a green box in Figure 5.1.

There is also considerable evidence that CLN may reduce oxidative stress as described in detail in Chapter 1, Section 1.6.3. Not only would prevention of oxidative stress-induced damage explain the protective effects of CLN against menadione-induced toxicity but as described detailed in Chapter 1, Section 1.4.3, Aβ-induced toxicity has been linked to oxidative stress and in particular SOD1 has

been found to be increased in cultures treated with Aβ (Pappolla *et al.* 1998). It was therefore hypothesised that the protective effects of CLN may be due to its antioxidant action. This hypothesis was examined by analysing the ability to CLN to prevent a menadione-induced increase in ROS production in B50 cells and the effects of bovine CLN, menadione and $A\beta_{1-42}$ on SOD1 protein levels in the primary hippocampal cultures described in Chapter 3 as well as B50 cells.

A further mechanism by which CLN may have a protective effect against Aβ-induced toxicity is via the release of cytokines. CLN has been demonstrated to be a modest inducer of cytokines, particularly of TNFα and gamma interferon (γIFN) (Inglot *et al.* 1996; Blach-Olszewska and Janusz 1997). As discussed in Chapter 1, Section 1.1.5.2 TNFα treatment has been found to reduce the toxicity of $A\beta_{1-42}$ by causing reduction in both protein levels and activity of Cdk5. Therefore a second hypothesis to be investigated was that CLN may reduce Cdk5 activation via the release of TNFα and thus prevent $A\beta_{1-42}$-induced toxicity.

As discussed in Chapter 1, Section 1.3.3 Aβ toxicity has been shown to involve the initiation of apoptosis, specifically caspase 3 activation, and menadione has also been demonstrated to induce caspase 3 activation (Adamec *et al.* 2000). Although CLN has been demonstrated to affect the cell cycle (Bacsi *et al.* 2005; Bacsi *et al.* 2007) and to down regulate gene expression of caspase 3 in the TR146 buccal mucosal cell line (Szaniszlo *et al.* 2009) no work has been done to investigate the effects of CLN on apoptosis in primary cells or to establish whether the change in caspase 3 gene expression correlates with changes in protein levels. The third hypothesis to examine was whether CLN may prevent caspase 3 activation.

The potential points for the mechanism of action of CLN against ROS production, alterations in anti-oxidant defence via changes in the levels of SOD1, prevention of Cdk5 activation via TNFα or prevention of the activation of caspase 3

and therefore apoptosis induction were investigated in this study and are shown in purple with question marks in Figure 5-1.

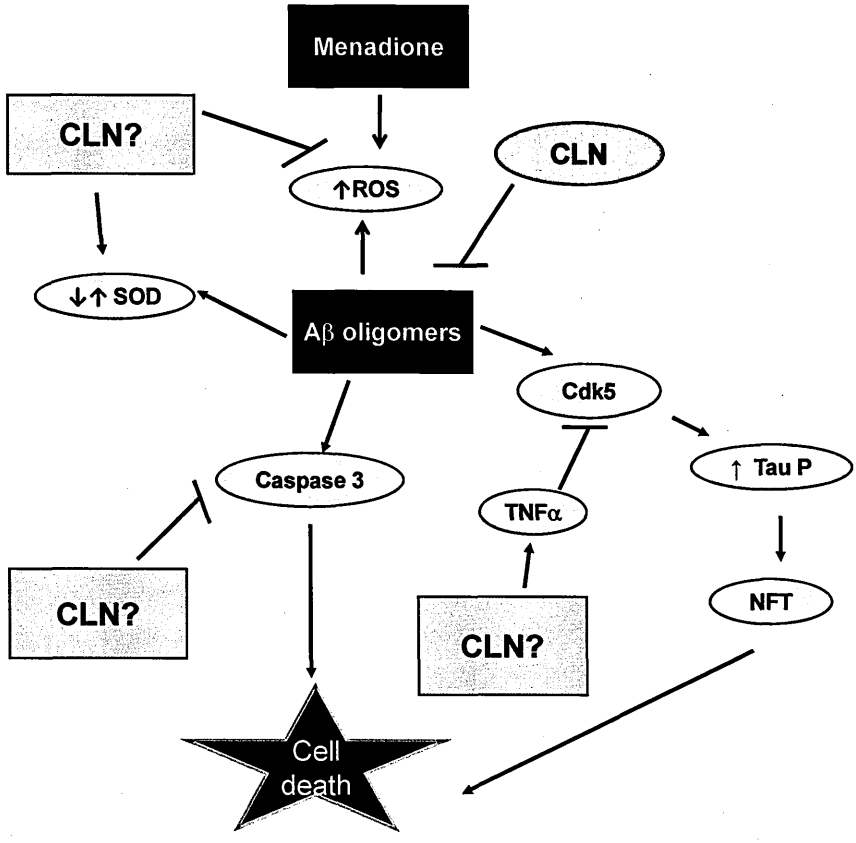


Figure 5-1: To show the known points of action for protection against Aβ-induced toxicity and potential mechanisms of action of CLN. Previously known points of action for CLN are in green and those investigated here are in purple with question marks. Toxic compounds are in black and compounds affected by these are in orange. Arrows represent an activation or increase, flat lines represent an inhibition.

5.3. Materials and methods

5.3.1. ROS production analysis

B50 cell cultures were plated at 100,000 cells/well in 6 well culture plates and treated *DIV*1 with 10μM menadione and 1μg/ml bovine CLN. On *DIV*2 ROS production analysis was carried out using the fluorescent ROS marker DCF and the images were analysed as described in Chapter 2, Section 2.11.

5.3.2. Western blotting

For these Western Blot experiments much higher cell densities were used than those used for experiments described in previous chapters. The rationale for this change was that whereas in previous experiments the key element had been to clearly observe changes in the cells morphology and number within the culures for these Western Blot experiments dense cultures were necessary to obtain the large amounts of protein that were needed from the cell lysate.

5.3.2.1. B50 cell lysate

B50 cells were plated at 100,000 cells/well in 6 well cultures plates and the media was changed *DIV*2.

5.3.2.2. Primary hippocampal cell lysate

Hippocampal cells were plated at 400,000 cells/well in 6 well culture plates and maintained as described in Chapter 2, Section 2.3.4.

5.3.2.3. Treatment

Bovine CLN, menadione and $A\beta_{1-42}$ were prepared as detailed in Chapter 2, Sections 2.5, 2.6 and 2.7 respectively. The cultures were treated on *DIV*3. For the menadione experiments cultures were treated with 5ng/ml or 50ng/ml bovine CLN and 1μM menadione in 0.001% ethanol and control cultures were treated with 0.001% ethanol in the culture media and bovine CLN was diluted in 0.001% ethanol in culture media to ensure that any changes observed were not due to the presence or absence of ethanol used to dissolve the menadione. For $A\beta_{1-42}$ experiments cultures were treated with i) PBS for control, ii) 10μM $A\beta_{1-42}$, iii) 5ng/ml or 5μg/ml bovine CLN and iv) $A\beta_{1-42}$ pre-incubated and co-administered with bovine CLN. The concentrations of CLN that have been chosen for this experiment, as for the experiment described in Chapter 4, Section 4.4.1.3 were based on the those used by Shuster *et al*, (2005) when investigating the effects of CLN against Aβ –induced toxicity. The concentration of $A\beta_{1-42}$ chosen here is lower than that used in the experiment described in Chapter 4, Section 4.4.1.3 because it was not desirable to cause severe cell loss for these experiments, only to initiate toxicity.

5.3.2.4. Western blotting

On *DIV*4 Western blot sample preparation, gels and Western blot analysis were carried out as described in Chapter 2, Section 2.12.

5.3.3. FACS analysis

Cells were plated in 6 well cultures plates as detailed in Chapter 2, Section 2.13 and the media was changed at *DIV*2. The cultures were treated on *DIV*3 with 5ng/ml bovine CLN and 1μM menadione.

On *DIV*4 FACS analysis of activated caspase 3 levels in the cultures was carried out as detailed in Chapter 2, Section 2.13.

5.4. Results

5.4.1. The effects of bovine CLN on the prevention of oxidative stress

5.4.1.1. The effect of CLN on menadione-induced ROS production in the B50 cell line

Twenty four hours of treatment of B50 cells with 10μM menadione was used to induce ROS production in order to produce a model in which to test the ability of CLN to reduce ROS production. The results from confocal microscopy of DCF stained cultures showed that 1μg/ml bovine CLN alone caused no change in ROS production compared to control cultures. However, 10μM menadione caused a large 20 fold increase (1,900±42% of controls. P= 0.037) in the level of fluorescence of the ROS marker DCF compared to control cultures (see Figure 5-2). Bovine CLN at 1μg/ml reversed this menadione-induced increase in ROS-induced fluorescence back to control levels (P = 0.046 see Figure 5-2).

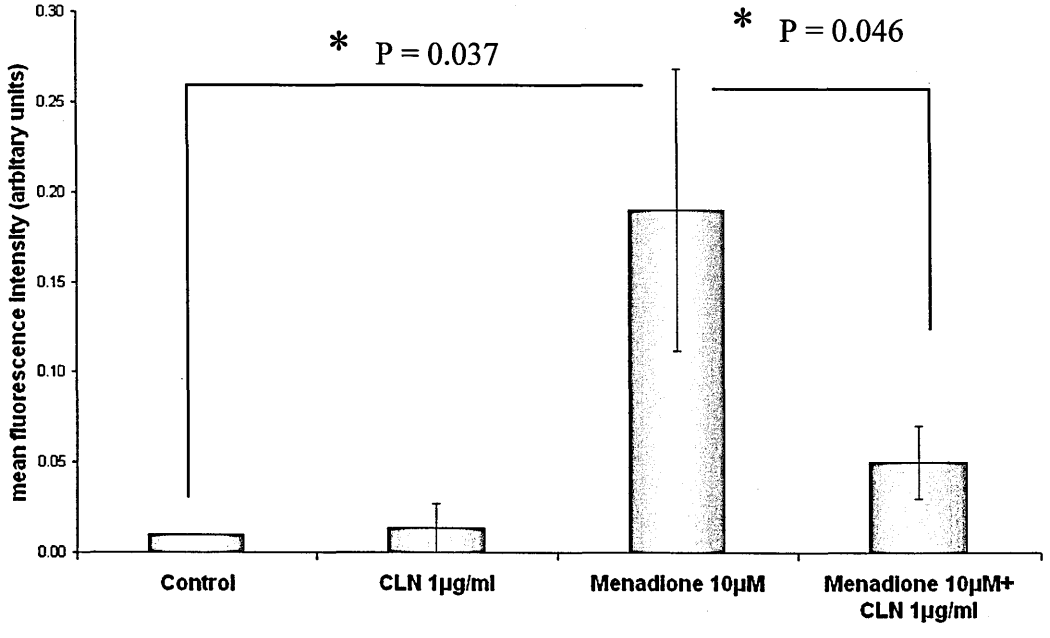


Figure 5-2: Quantification of DCF fluorescence on B50 cell line cultures from confocal images showing that CLN alone had no effect on ROS production compared to controls (p = 0.480) but ROS levels were increased upon treatment with 10μM menadione (P = 0.037). This menadione-induced increase was reversed when CLN is given along with menadione (P = 0.046). The ROS production was not changed in cultures treated with menadione and CLN compared to controls (P = 0.114) or CLN treated cultures (P = 0.116). n=3 and error bars represent standard errors. * represents p< 0.05.

5.4.1.2. The effect of treatment with bovine CLN and beta-amyloid on the protein levels of the antioxidant enzyme SOD1 in primary hippocampal cultures.

Treatment of the primary hippocampal cultures with bovine CLN alone at 5ng/ml or 5µg/ml did not lead to changes in the protein levels of SOD1 compared to controls. However 24 hour treatment of these cultures with 10µM $A\beta_{1-42}$ did cause an increase in the SOD1 protein levels of 29±8% compared to the control (P = 0.037, see Figure 5-3 and 5-4). Pre-incubation and co-administration of 5ng/ml or 5µg/ml bovine CLN with $A\beta_{1-42}$ reduced the $A\beta_{1-42}$-induced increase in SOD1 protein levels to control levels and densitometry showed this reduction to just reach significance (P = 0.05, see Figures 5-3 and 5-4).

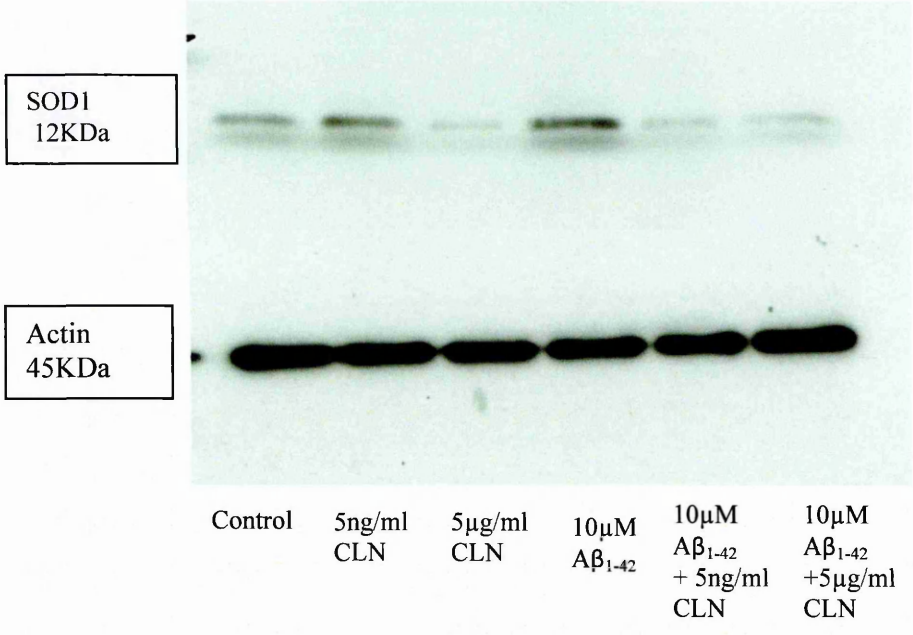


Figure 5-3: A representative Western blot for SOD1 on primary hippocampal cell lysate after treatment with bovine CLN and $A\beta_{1-42}$ showing that 10µM $A\beta_{1-42}$ caused an increase in SOD1 protein levels but this $A\beta_{1-42}$-induced increase was reversed when CLN at 5ng/ml or 5µg/ml was pre-incubated and co-administered with $A\beta_{1-42}$; actin is shown for normalization.

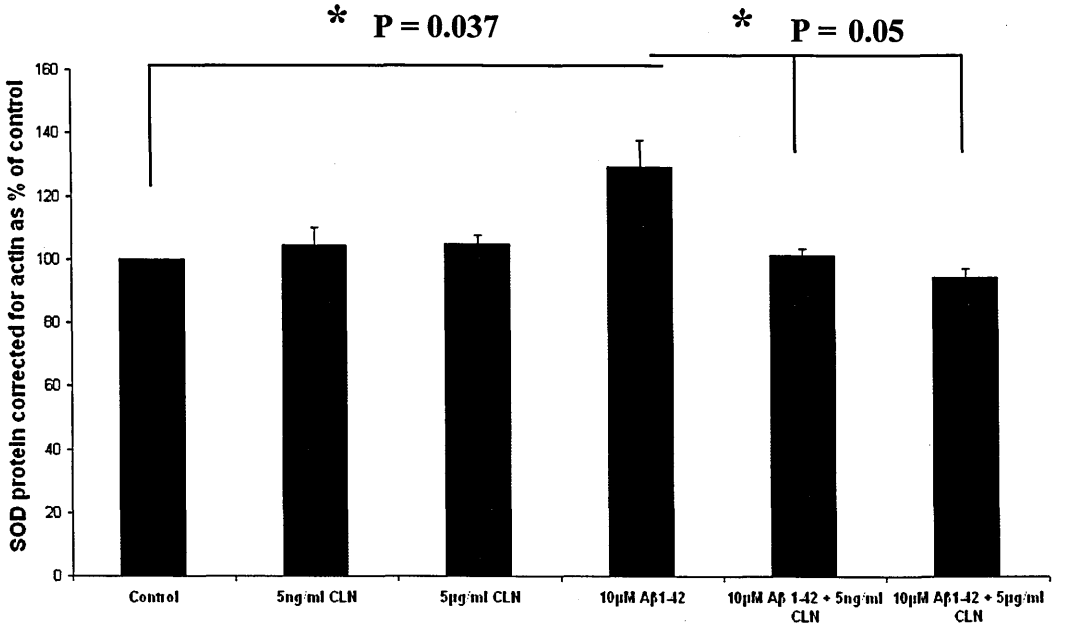


Figure 5-4: Densitometric quantification of Western blots for SOD1 from primary hippocampal cell line lysate after treatment with 5ng/ml or 5µg/ml bovine CLN, 10µM $A\beta_{1-42}$ or CLN pre-incubated and co-administered with $A\beta_{1-42}$. SOD1 levels in cultures treated with CLN at 5ng/ml or 5µg/ml did not differ from controls (P = 0.487 and 0.121 respectively). 10µM $A\beta_{1-42}$ increased SOD1 protein levels in these cells (P = 0.037) and pre-incubation and co-administration of bovine CLN prevented this increase (P = 0.05). n=3. * represents $P < 0.05$. Error bars represent standard errors.

5.4.1.3. The effect of treatment with bovine CLN and beta-amyloid on the protein levels of the antioxidant enzyme SOD1 in B50 cells.

Treatment of B50 cell cultures with 5ng/ml bovine CLN caused an 80±7% increase in the protein levels of SOD1 compared to control (P = 0.037, see Figures 5-5 and 5-6). Treatment with 10µM $A\beta_{1-42}$ led to a small decrease in the levels of SOD1 protein in these cultures (P = 0.037) and the CLN mediated increase in protein levels was absent when CLN was pre-incubated and co-administered with 10µM $A\beta_{1-42}$ (see Figures 5-5 and 5-6).

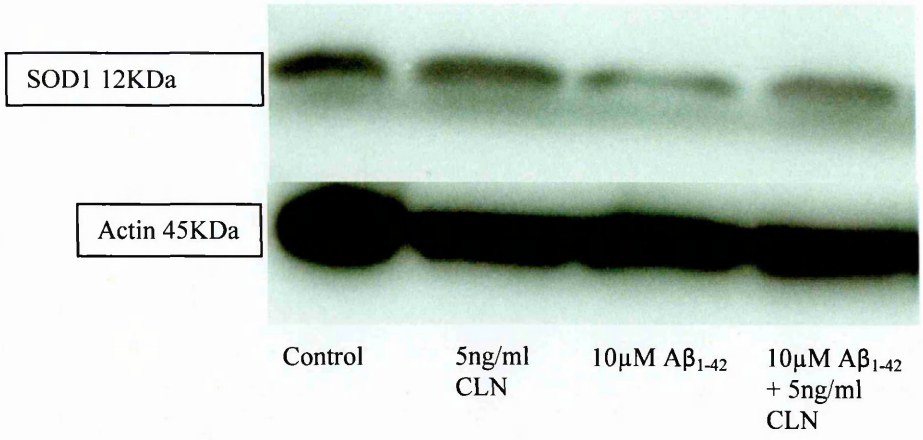


Figure 5-5: A representative Western blot for SOD1 on B50 cell lysate after treatment with 5ng/ml bovine CLN and 10μM $A\beta_{1-42}$ showing an increase in SOD1 protein levels upon treatment with 5ng/ml CLN compared to control and a small decrease with $A\beta_{1-42}$ compared to control. Actin is shown for normalization.

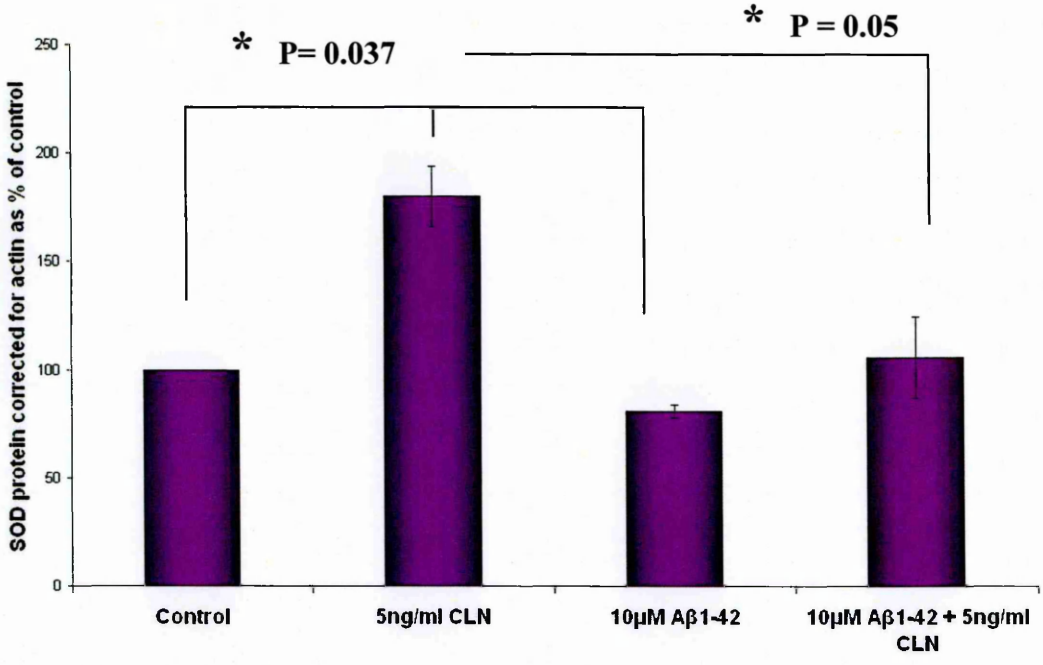


Figure 5-6: Densitometric quantification of Western blots for SOD1 on B50 cell line lysate after treatment with 5ng/ml bovine CLN, 10μM $A\beta_{1-42}$ or CLN pre-incubated and co-administered with $A\beta_{1-42}$ showing that bovine CLN increased SOD1 protein levels in these cells (P = 0.037) but 10μM $A\beta_{1-42}$ caused only a small decrease (P = 0.037) in the enzyme protein levels. When CLN was pre-incubated and co-administered with $A\beta_{1-42}$ the protein levels were reduced form those observed with CLN alone (P = 0.05). n=3. * represents $P < 0.05$. Error bars represent standard errors.

5.4.1.4. The effect of treatment with bovine CLN and menadione on the protein levels of the antioxidant enzyme SOD1 in B50 cells.

Both bovine CLN and menadione at the concentrations given were found to cause a small increase in the SOD1 protein levels in cultures and this increase was slightly more pronounced when CLN and menadione were given together (see Figure 5-7). Densitometric analysis showed that none of these changes reached significance (see Figure 5-8).

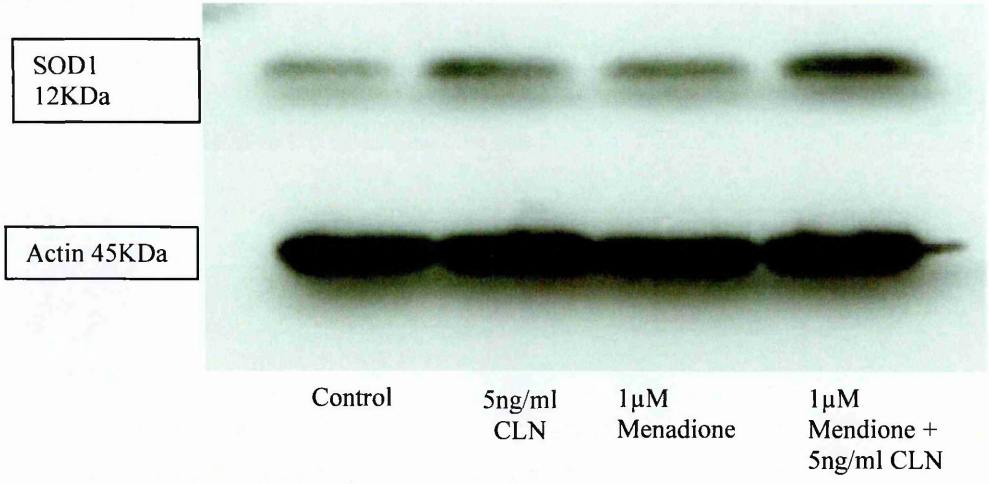


Figure 5-7: A representative Western blot for SOD1 on B50 cell line lysate after treatment with 5ng/ml bovine CLN, 1μM menadione or both showing that both treatments increased SOD1 protein levels in these cells but SOD1 protein levels were highest when CLN and menadione were given together. Actin is shown for normalisation.

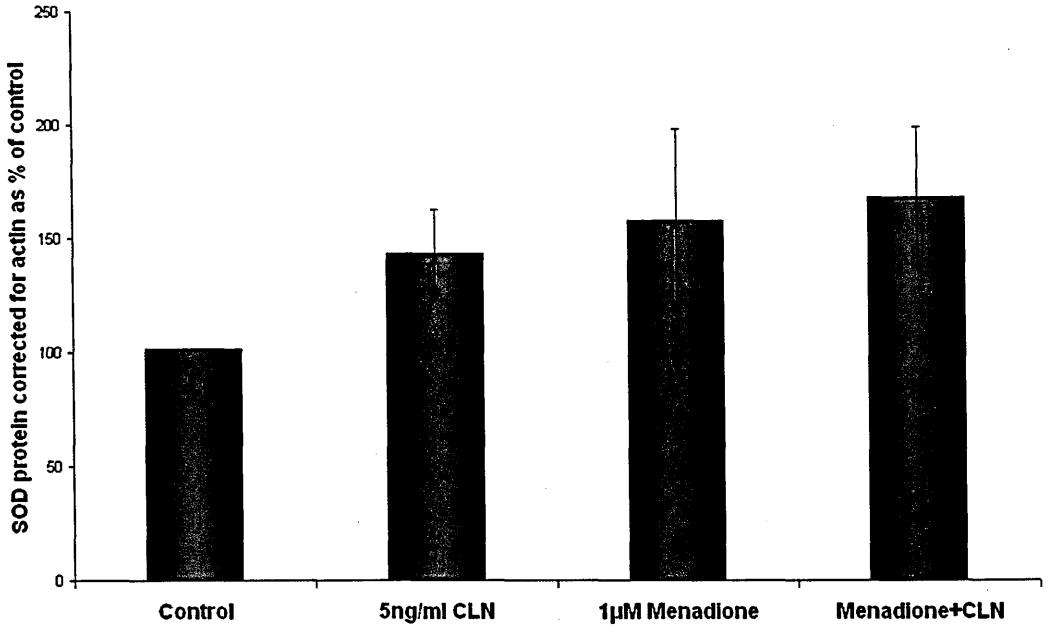


Figure 5-8: Densitometric quantification of Western blots for SOD1 on B50 cell line lysate after treatment with 5ng/ml bovine CLN, 1μM menadione or both showing that both treatments increased SOD1 protein levels in these cells but SOD1 protein levels were highest when CLN and menadione were given together. n=4. Error bars represent standard errors. From a Kruskal-Wallis test this experiment did not reach significance (P = 0.270).

5.4.2. The effect of bovine CLN on Cdk5 levels

5.4.2.1. The effect of treatment with CLN and beta-amyloid on the protein levels of Cdk5 in primary hippocampal cultures.

A small increase in Cdk5 protein levels was observed upon treatment with CLN or 10μM $A\beta_{1-42}$ alone. This increase in Cdk5 protein levels over control levels was significant for CLN at 5ng/ml and 5μg/ml as well as for 10μM $A\beta_{1-42}$ (P= 0.037.see Figures 5-9 and 5-10). Pre-incubation and co-administration of 5ng/ml CLN with $A\beta_{1-42}$ led to significantly higher Cdk5 levels compared to controls (P= 0.037) although the effects did not appear to be cumulative. However, pre-incubation and co-administration of 5μg/ml CLN with $A\beta_{1-42}$ resulted in Cdk5 protein levels that were similar to control levels and levels that were significantly lower than those observed with $A\beta_{1-42}$ treatment alone (P = 0.046) but not CLN treatment alone (see Figures 5-9 and 5-10).

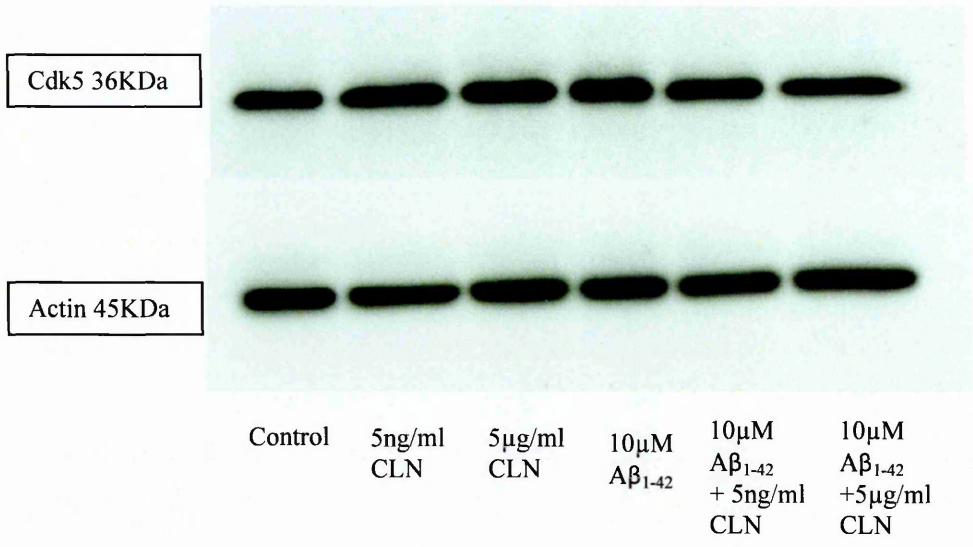


Figure 5-9: A representative Western blot for Cdk5 on primary hippocampal cell lyase after treatment with bovine CLN and 10µM $A\beta_{1-42}$ showing that CLN and $A\beta_{1-42}$ both led to an increase in Cdk5 protein levels but when 5µg/ml CLN was pre-incubated and co-administered with $A\beta_{1-42}$ Cdk5 protein levels were at control levels. Actin is shown for normalisation.

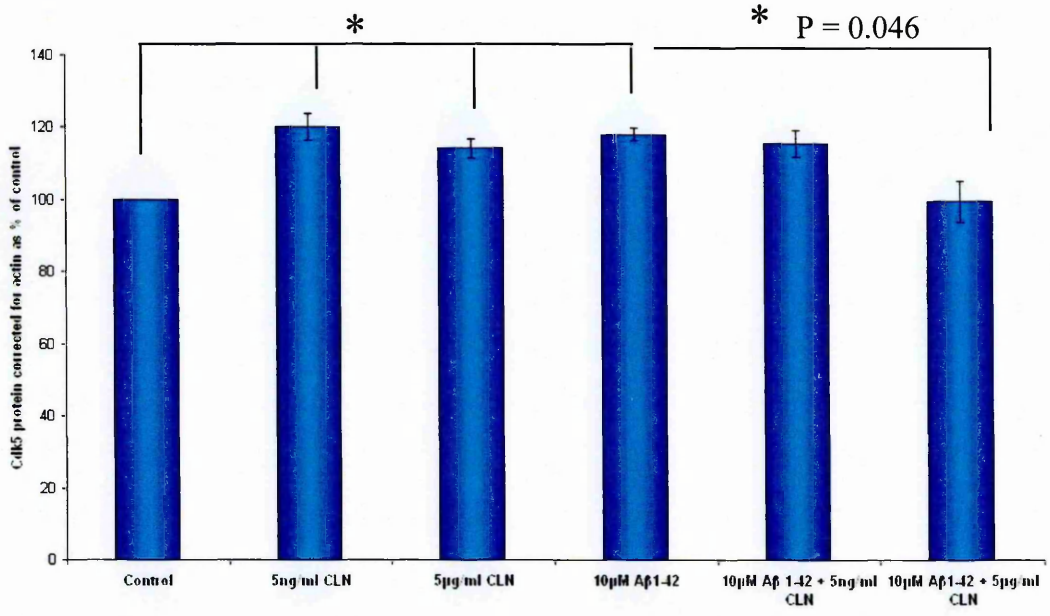


Figure 5-10: Densitometric quantification of Western blots for Cdk5 on primary hippocampal cell lysate after treatment with CLN and Aβ showing that CLN and $A\beta_{1-42}$ both led to an increase in Cdk5 protein levels compared to controls (P = 0.037) and this increase remained when 5ng/ml CLN was pre-incubated and co-administered with $A\beta_{1-42}$ (P = 0.037). However when 5µg/ml CLN was pre-incubated and co-administered with $A\beta_{1-42}$ Cdk5 protein levels were reduced compared to $A\beta_{1-42}$ alone (P = 0.046). n=3. * represents $P < 0.05$. Error bars represent standard errors.

5.4.2.2. The effect of treatment with CLN and beta-amyloid on the protein levels of Cdk5 in B50 cell cultures.

Some variability in the Cdk5 protein levels was observed when B50 cultures were treated with 10μM $A\beta_{1-42}$, 5ng/ml bovine CLN or 10μM $A\beta_{1-42}$ pre-incubated and co-administered with CLN. However, overall there was no notable change in the protein levels of Cdk5 in B50 cultures with any of these treatments when compared to controls although there was a small decrease with $A\beta_{1-42}$ treatment alone (see Figures 5-11 and 5-12).

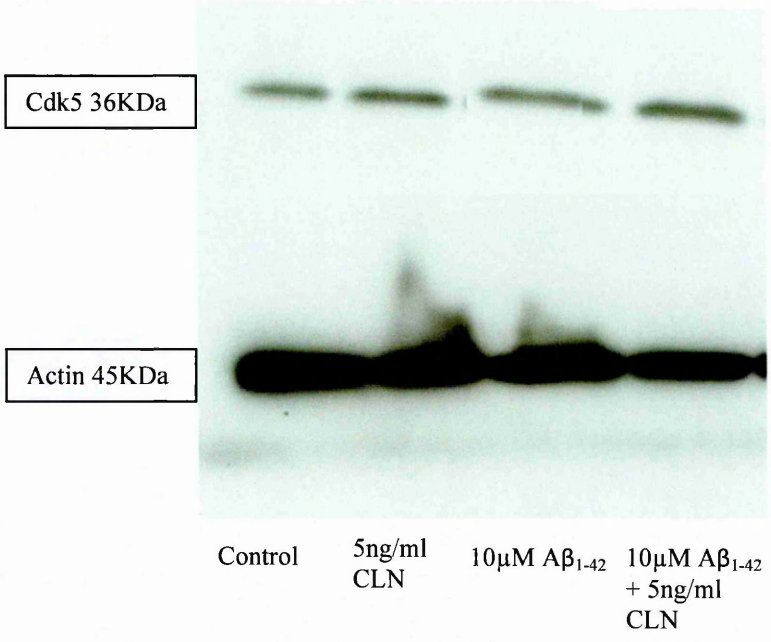


Figure 5-11: A representative Western blot for Cdk5 on B50 cell line lysate after treatment with bovine CLN and 10μM $A\beta_{1-42}$ showing little change from controls with any treatment. Actin is shown for normalisation.

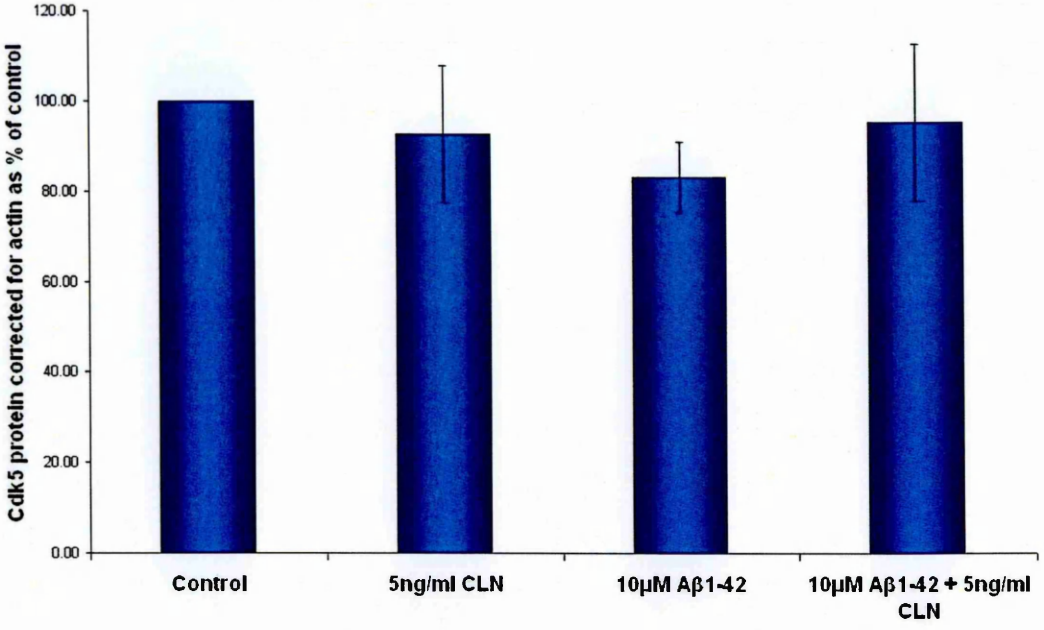


Figure 5-12: Densitometric quantification of Western blots for Cdk5 on B50 cell lysate after treatment with 5ng/ml bovine CLN and 10μM $A\beta_{1-42}$ showing little change in Cdk5 protein levels with CLN or $A\beta_{1-42}$ treatment. n=3. Error bars represent standard errors. From a Kruskal-Wallis test this experiment did not reach significance (P = 0.501).

5.4.3. The effect of bovine CLN on the number of cells expressing activated caspase 3

FACS analysis showed that there was variation in background number of cells expressing active caspase 3 in control cultures. However, no consistent increase or decrease in the percentage of cells expresing active caspase 3 compared to controls in response to CLN was seen in either primary hippocampal cells (see Figures 5-13 and 5-14) or B50 cells (see Figures 5-15 and 5-16). In B50 cells 1μM menadione caused a reduction in the percentage of cells expressing active caspase 3 compared to controls although upon quantification (see Figures 5-15 and 5-16) this reduction in expression did not reach significance.

A: Control **B: 50ng/ml CLN**

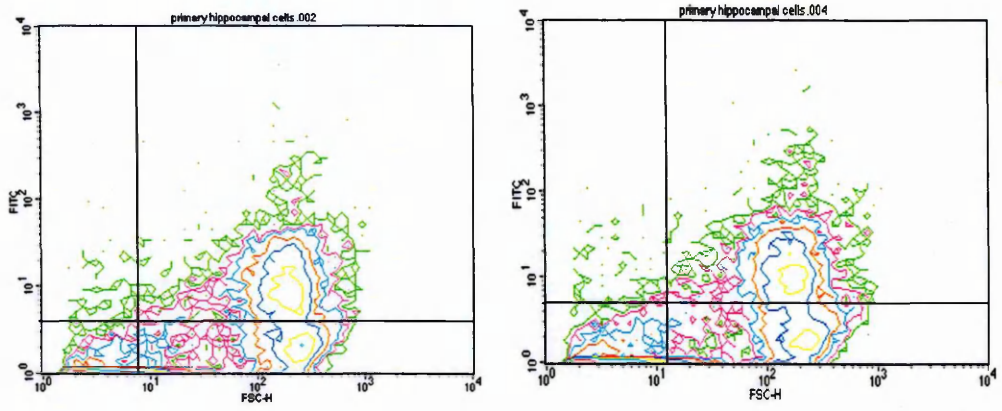


Figure 5-13: FACS analysis contour plots for active caspase 3 in primary hippocampal cells after treatment with 50ng/ml bovine CLN. The upper right quadrant represents cells in the cell population of interest and are positively stained for activated caspase 3.The bottom right quadrant represents cells in the cell population of interest but that are unstained for caspase 3 and the left quandrants represent cells not in the population of interest or cell debris. CLN did not consistently cause a change in the number of cells expressing activated caspase 3.

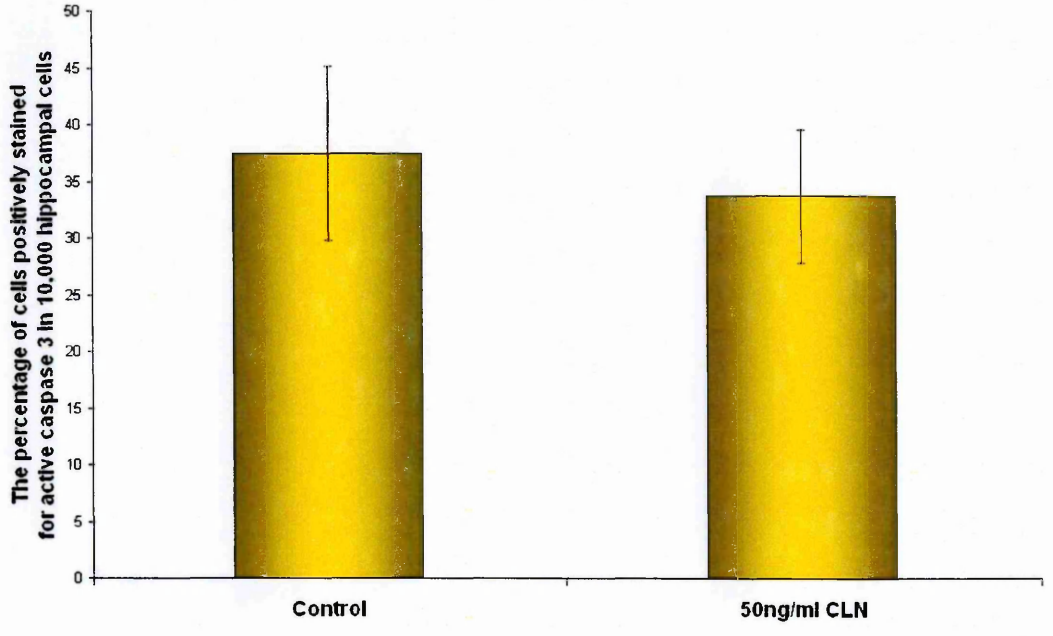


Figure 5-14: Quantification of FACS analysis for active caspase 3 in primary hippocampal cells after treatment with 50ng/ml bovine CLN. CLN did not consistently cause a change in the number of cells expressing activated caspase 3 compared to control cultures (from one-way ANOVA P = 0.878). n=6 Error bars represent standard errors.

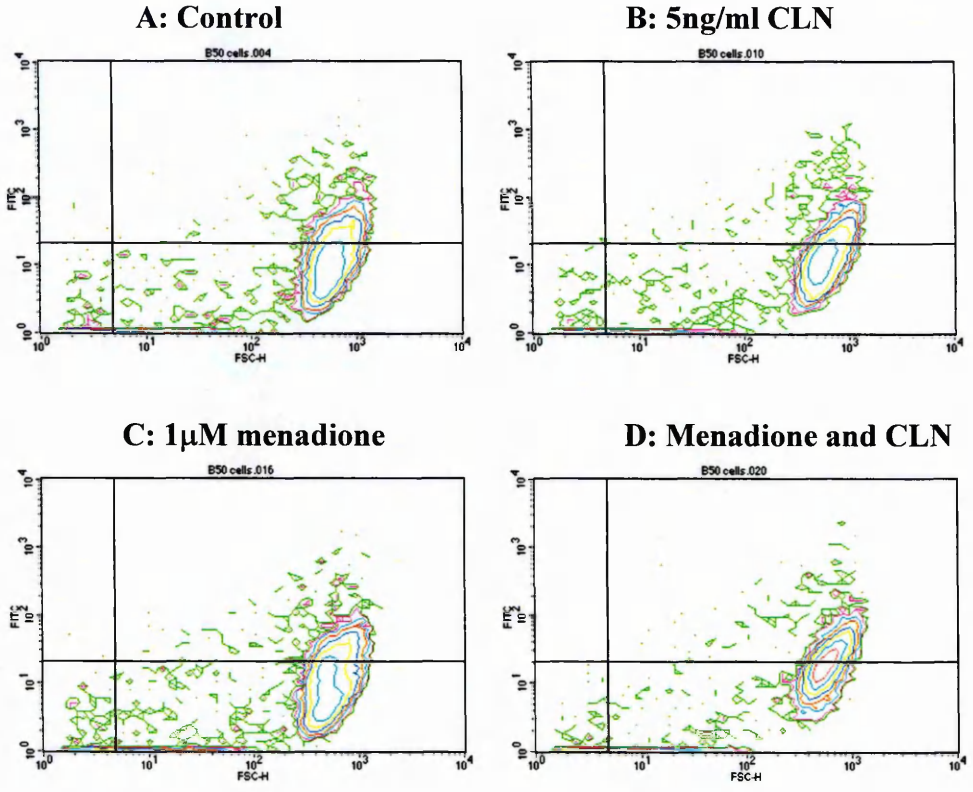


Figure 5-15: FACS analysis contour plots for active caspase 3 in B50 cells after treatment with 5ng/ml bovine CLN and 1μM menadione. The upper right quadrant represents cells that in the cell population of interest and are positively stained for activated caspase 3.The bottom right quadrant represents cells in the cell population of interest but that are unstained for caspase 3 and the left quandrants represent cells not in the population of interest or cell debris. There was no consistent change in the number of cells expressing activated caspase 3 upon treatment with CLN but menadione treatment caused a small reduction in the number of cells expressing activated caspase 3.

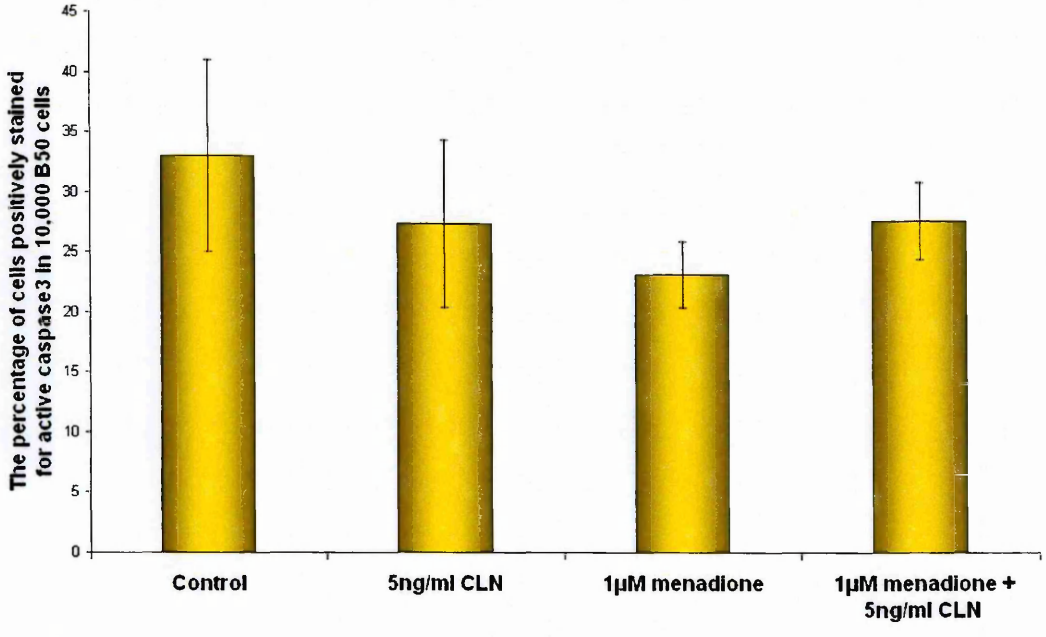


Figure 5-16: Quantification of FACS analysis for active caspase 3 in B50 cells after treatment with 5ng/ml bovine CLN and 1μM menadione showing that there was no consistent change in activated caspase 3 expression levels upon treatment with CLN. Menadione treatment caused a small reduction in the number of cells expressing activated caspase 3 although a one-way ANOVA showed that these did not reach significance (P = 0.494). n=3. Error bars represent standard errors.

5.5. Discussion

Having investigated potential protective effects of bovine CLN against menadione and $A\beta_{1-42}$-mediated toxicity in primary hippocampal cells the next stage of the project was to investigate, using the same cell culture systems, how CLN may affect anti-oxidant defence, protein kinase protein levels and apoptosis.

5.5.1. The effect of bovine CLN, menadione and beta-amyloid on oxidative stress

In accordance with previous studies demonstrating the ability of CLN to reduce H_2O_2 or Aβ-induced increases in ROS (Boldogh and Kruzel 2008) CLN was found to prevent a menadione-induced increase in ROS in B50 cells.

The results of the Western blot experiment which examined changes in SOD1 protein levels in primary hippocampal cultures treated with bovine CLN at 5ng/ml or 5μg/ml and 10μM $A\beta_{1-42}$ showed that the protein levels were unchanged with CLN treatment alone but increased by 29±8% compared to controls with $A\beta_{1-42}$ treatment alone. This $A\beta_{1-42}$-mediated increase in the Cu/Zn form of the SOD protein levels is in agreement with previous experiments by Pappolla *et al.* (1998) studying SOD1 levels, which showed that SOD1 is increased in a transgenic mouse model of AD as well as in PC12 cell cultures that had been treated with $A\beta_{25-35}$. Furthermore it was found that when bovine CLN at both 5ng/ml and the high concentration of 5μg/ml was pre-incubated and co-administered with $A\beta_{1-42}$ the $A\beta_{1-42}$-induced increase in SOD1 protein levels were reduced to control level. This result implies that in primary hippocampal cells bovine CLN at the concentrations tested is able to prevent a $A\beta_{1-42}$-induced increase in oxidative stress which leads to increased expression of antioxidant defences.

However, the same experimental conditions in the B50 cell line produced different results to those in the primary hippocampal cells. In the B50 cell line 5ng/ml bovine CLN caused an 80±7% increase in SOD1 protein levels compared to controls with a small decrease in protein levels in response to $A\beta_{1-42}$ treatment and no change compared to controls when CLN was pre-incubated and co-administered with $A\beta_{1-42}$. The mechanism of action of CLN and $A\beta_{1-42}$ must therefore differ between primary hippocampal cells and B50 cells. Abromov and Duchen (2005) showed that the $A\beta_{1-42}$-mediated increase in oxidative stress in primary cortical and hippocampal cultures was mediated via depolarization in astrocytes present in the cultures (Abramov *et al.* 2005) so the lack of SOD1 response to $A\beta_{1-42}$ in B50 cells could be due to the absence of astrocytes in these cultures. Furthermore, as discussed in Chapter 4, Section 4.5, the culture media used may affect the survival of cells in culture (Xie *et al.* 2000) and this may therefore have an effect on the cell type specific response to $A\beta_{1-42}$.

As in the Western blot experiments described above investigating the effect of bovine CLN with $A\beta_{1-42}$ on B50 cells CLN alone was found to increase in SOD1 protein levels in B50 cells in Western blot experiments analysing the effects of CLN and menadione on SOD1 protein levels. In these experiments bovine CLN or menadione alone induced a small increase in SOD1 protein levels and these increase appeared to be cumulative. There are two possible explanations for the increase in SOD1 protein levels in B50 cells in response to CLN; firstly it could be that in this cell line CLN is inducing oxidative stress and the related damage which is leading to the induction of antioxidant defence or secondly CLN may be protective and directly induce the expression of the antioxidant enzyme in this cell line without the need for stress induction in a similar manner to that where oestrogens have been shown to induce the expression of SOD2 in MCF-7 cells (Borras *et al.* 2005). The latter

hypothesis is supported by the fact that CLN was not found to increase ROS in the B50 cells using the ROS marker DCF and experimental evidence so far indicates that CLN is an antioxidant in its effect (Boldogh *et al.* 2003; Bacsi *et al.* 2006). However, the concentration of CLN used in the ROS production experiment was different to that used in the present experiment and previous work has been done using different cells. These results coupled with the finding that CLN was able to reduce a menadione-induced increase in ROS in the B50 cell line strongly suggest that CLN may have a protective effect against menadione and $A\beta_{1-42}$ by reducing oxidative stress. The finding that bovine CLN is able to affect the protein levels of SOD1 in both primary hippocampal cells and B50 cells is in keeping with work by Szaniszlo *et al.* (2009) which showed that CLN is able to alter the expression of SOD in a microarray analysis of the buccal mucosal cell line.

5.5.2. The effect of bovine CLN and beta-amyloid on the protein levels of Cdk5

The second part of the work discussed in this Chapter analysed the protein levels of Cdk5 in hippocampal and B50 cell cultures in response to bovine CLN and $A\beta_{1-42}$. Experiments on B50 cells showed that CLN alone or pre-incubated and co-administered with $A\beta_{1-42}$ did not cause a decrease in Cdk5 levels. In primary hippocampal cultures CLN treatment alone led to an increase in CDK5 protein levels which was significant. This result apparently argues against the hypothesis that CLN may have a protective effect against $A\beta_{1-42}$-induced toxicity by causing the release of TNFα which would decrease the protein levels of Cdk5 and therefore prevent $A\beta_{1-42}$ mediated Tau phosphorylation. This apparent lack of effect of CLN on Cdk5 may be due to the fact that only protein levels were examined. There may have been a change Cdk5 activity that could not be detected in these experiments but would be observed if a Cdk5 activity assay was employed. There was also a significant

increase seen in Cdk5 protein levels in primary hippocampal cells upon treatment with $A\beta_{1-42}$ which was reversed with the higher concentration of CLN (5μg/ml) and there may therefore be a small effect of CLN on $A\beta_{1-42}$-induced Cdk5 possibly via TNFα.

5.5.3. The effect of bovine CLN on the number of cells expressing activated caspase 3

The results of FACS analysis used to determine the effects of bovine CLN on activation of the apoptotic initiator, caspase 3, showed that CLN alone had no consistent effect on caspase 3 activation compared to controls in either primary hippocampal cells or B50 cells. The variation encountered in the expression of active caspase 3 may be due to natural background variation in the levels of apoptosis within the cultures. Microarray analysis work carried out by Szaniszlo *et al.* (2009), on TR146 buccal mucosal cells showed that 100ng/ml bovine CLN down-regulated gene expression of caspase 3. However this study used a different cell type and the longest time point studied in the previous work was 18 hours compared to the 24 hour treatment prior to FACS analysis. FACS analysis also showed active caspase 3 expression to be relatively unchanged compared to controls in primary hippocampal cells and decreased in B50 cell line upon treatment with 1μM menadione for 24 hours. These conditions for menadione treatment have previously been demonstrated to lead to the induction of apoptosis and increased expression of active caspase 3 in primary hippocamapal cells (Adamec *et al.* 2000). However Ibuki *et al.* (2006), found that menadione could prevent the apoptosis induced by serum depletion and cell detachment in the NIH-3T3 cell line which may explain this decrease in active caspase 3 expression observed in the cell line. One possible explanation for the lack of a menadione-induced increase in active caspase 3 in the hippocampal cultures is

that Adamec *et al.* (2000), used hippocampal cultures at three weeks in culture and for these present experiments 3 day old cultures were used for consistency with the immunocytochemistry, the toxicity experiments described in Chapter 4 and the Western blot experiments detailed in this Chapter, Sections, 5.4.1 and 5.4.2.

5.5.4. Summary

These results show that bovine CLN can prevent ROS production and alter the protein levels of the antioxidant enzyme SOD1. CLN does not however have significant effects on reducing the protein levels of the kinase Cdk5 or activation of caspase 3. The effects of CLN on SOD1 protein levels differed between the two culture systems used in this study which may partially explain the lack of protective effects of CLN against menadione-induced toxicity seen in the B50 cultures. The small changes in the number of MAP-2 positive neurons seen in hippocampal cultures when CLN was co-adminsitered with on menadione or $A\beta_{1-42}$ comapred to treatment with either of these compounds alone therefore appear to be largely due to antioxidant properties of CLN.

Chapter 6

General discussion

6.1. Summary of findings

AD is the most common form of dementia and there is, at present, no cure for the disease although treatment can be given to slow its progression and make patients more comfortable. The major cause of the neuronal loss and subsequent cognitive decline in AD patients is the accumulation of extracellular plaques of Aβ which is produced from cleavage of APP, and intracellular NFT containing Tau. It is now widely accepted that the amyloid plaques are the initiating factor in neurodegeneration, causing toxicity *in vivo* and *in vitro*, and leading to the formation of NFT which will in turn cause further neurodegeneration. Therefore compounds which can prevent the accumulation, aggregation or toxicity of Aβ at any level of this process may potentially lead to the development of therapies to improve the prognosis for AD patients and slow disease progression.

CLN has been found to stabilise symptoms of disease in patients with AD in clinical trials lasting up to 16 months. It has also been demonstrated to improve spatial memory in aged rats (Popik *et al.* 2001) and passive avoidance learning in the day old chick (Stewart and Banks 2006). *In vitro* CLN has been shown to have an antioxidant effect (Boldogh *et al.* 2003) and to prevent the aggregation of Aβ (Schuster *et al.* 2005). However, the mechanism of action of CLN that allows the beneficial effects that have been found *in vivo* is still unknown and only one study has been carried out to look directly at the effects of CLN on Aβ-mediated toxicity in culture (Schuster *et al.* 2005). Another study has investigated gene expression changes relating to Aβ (Szaniszlo *et al.* 2009) but neither of these studies were carried out in primary cells and they did not examine cell type specific changes.

The aim of this project was therefore to investigate the mechanism of action of CLN. Previous research has involved only ovine CLN so here the effects of

bovine CLN on primary hippocampal cells in culture have been examined for the first time. The ability of bovine CLN to prevent $A\beta_{1-42}$-induced and menadione-induced effects on these cells and on B50 cells were also analysed.

The first part of the research analysed the effects of bovine CLN on hippocampal cells in culture when given alone, and also possible protective effects against menadione-induced, oxidative stress mediated, or $A\beta_{1-42}$-induced toxicity. This work demonstrated that bovine CLN at 100ng/ml has a small trophic effect on neurons and astrocytes in low density cultures of primary hippocampal cells from E18 hippocampus (see Figure 4-12). Furthermore, this concentration of CLN was found to cause a trend towards a small reduction in the effects of menadione-induced toxicity in primary hippocampal cells in culture (see Figures 4-6 and 4-12) and it did not protect B50 cells against menadione-induced toxicity (see Figures 4-19 to 4-23). A higher concentration (5μg/ml) of bovine CLN appeared to slightly reduce $A\beta_{1-42}$-induced toxicity although these effects seen in hippocampal cells did not reach significance (see Figure 4-28). However, a study carried out as part of this work using 5ng/ml bovine CLN demonstrated a significant protective effect against $A\beta_{1-42}$-induced toxicity. This result is in keeping with the findings of Schuster *et al.* (2005), who found that there was a negative correlation between the protective effect on cell survival that they observed and the concentration of CLN that was used. These authors found that in SH-SY5Y cells there was significant protection against $A\beta_{1-40}$-induced toxicity when CLN was pre-incubated and co-administered with the $A\beta_{1-40}$ (Schuster *et al.* 2005). 0.25μM CLN was seen to give about 30% cell survival but this protective effect increased to over 40% cell survival at 0.0025μM CLN (Schuster *et al.* 2005). This preliminary experiment using CLN at 5ng/ml described here therefore needs to be repeated but was beyond the means of the present research due to finance and time constraints.

The next area of analysis was to investigate the specific mechanism of action of CLN. On studying the antioxidant effects of bovine CLN it was found that bovine CLN could prevent a menadione-induced increase in ROS in B50 cells (see Figure 5-2). Furthermore bovine CLN at either 5ng/ml or 5μg/ml was able to prevent $A\beta_{1-42}$-induced increase in SOD1 protein levels in primary hippocampal cells demonstrating an antioxidant effect (see Figures 5-3 and 5-4). This corroborates previous work discussed in Chapter 1, Section 1.6.3, demonstrating antioxidant effects of CLN in several cell types and shows for the first time that the effect is present in primary hippocampal cells. In B50 cells CLN at 5ng/ml caused an increase in SOD1 (see Figures 5-5 and 5-6) implying that CLN increases the antioxidant defence in these cells; however, the full details of the mechanism of this effect would require further investigation.

It has been reported in the literature that TNFα-mediated cell protection against $A\beta_{1-42}$-induced toxicity coincides with decreased protein levels of Cdk5 (Orellana *et al.* 2006) and CLN has been found *in vitro* and *in vivo* to cause the release of TNFα (Inglot *et al.* 1996; Blach-Olszewska and Janusz 1997). The experiments carried out here to analyse the effect of CLN on Cdk5 protein levels showed that bovine CLN at 5μg/ml did prevent an $A\beta_{1-42}$-induced increase in Cdk5 levels (see Figures 5-9 and 5-10). However, bovine CLN alone also caused an increase in Cdk5 protein levels implying that CLN does not mediate cellular protection via the release of TNFα inducing a decrease in Cdk5 (see Figures 5-9 and 5-10).

FACS analysis of the percentage of cells expressing activated caspase 3 in primary hippocampal cultures and B50 cells showed that CLN did not have a consistent effect on the level of expression of activated caspase 3 in either cell type compared to control cultures (see Figures 5-13 to 5-16). However, CLN may affect

the activation of a different apoptosis initiator and the effect of CLN on Aβ-induced changes in activated caspase 3 expression was not analysed.

6.2. CLN as a protective agent against beta-amyloid induced toxicity

As detailed in Chapter 1 and summarised in Chapter 5, Section 5.2.2 there are many mechanisms by which a compound may exert a protective effect against Aβ-induced toxicity *in vivo* or *in vitro*.

The findings of the work presented here are summarised in Figure 6-1 which illustrates the points in the mechanism of Aβ-induced toxicity that CLN affects. The results imply that the protective effects of CLN *in vitro* and benefits *in vivo* are mediated largely by antioxidant effects of CLN and possibly by decreasing an $A\beta_{1-42}$-induced increase in Cdk5 but are unlikely to be mediated by preventing the initiation of apoptosis, at least at the level of caspase 3 activation, or by decreasing basal Cdk5 levels.

This work along with previous studies showing CLN to have anti-oxidant effects (reviewed in Stewart 2008), to be able to reduce the aggregation of $A\beta_{1-40}$ (Schuster *et al.* 2005) and to decrease c-JUN activation (Szaniszlo *et al.* 2009) demonstrates that CLN has effects on several of the mechanisms of Aβ-induced toxicity. Several other compounds have also been shown to mediate a protective effect against Aβ via effects on SOD levels or activity, such as Neurokinin B (Mantha *et al.* 2006), oestrogen (Borras *et al.* 2005; Dong *et al.* 2007) and hyperzine A (Xiao *et al.* 2000).

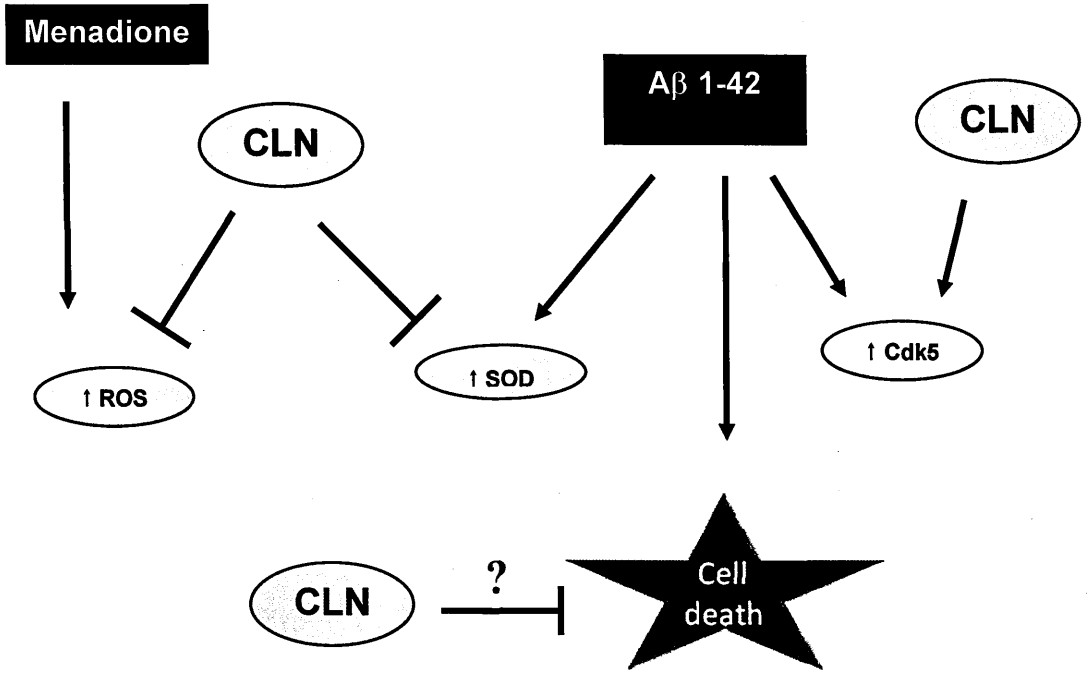


Figure 6-1: Diagrammatic illustration of the points in the mechanism of Aβ-induced toxicity that CLN has been found to affect and those on which it may not have any effect. The major effects were found to be on ROS production and SOD1 with a small effect on Cdk5.

6.3. Potential limitations

6.3.1. The effect of culture on cells

This study has used primary rat hippocampal cultures and B50 neuronal cells as model systems for investigating the mechanism of action of CLN and has added to the knowledge of the mechanism of action of CLN and, in particular, has illuminated its anti-oxidant effects. There are very valid reasons for using culture systems as models and the rationale for using hippocampal cultures in this study is discussed in detail in Chapter 3, Section 3.2.1.

However, a 2D culture can never be perfect due to the lack of a natural 3D environment without the trophic factors and cellular interactions which occur *in vivo* as well as the additional stress on the cells from dissociation and culturing. Halliwell discussed the possible extent of this 'culture shock' and the influence of oxidative stress on this phenomenon in detail (Halliwell 2003).

Culturing cells can also alter their development compared to that which occurs during *in vivo* development. For example, in long-term culture of embryonic stem cells, changes in proliferation and differentiation have been found to occur using the cell proliferation marker 5-bromo2'-deoxyuridine (BrdU) (Park *et al.* 2008). Although Banker and Cowen (1979) saw that some neurons in culture appeared to resemble normal pyramidal cells when they compared hippocampal neurons from E19 rats that had been in culture for 7 days to hippocampal neurons from P4 rats, they showed that those neurons which had been kept *in vitro* had shorter dendrites and were less advanced than those that had the same developmental time but had developed *in vivo* (Banker and Cowan 1979). Moreover, these authors found that the axons which were seen after longer periods *in vitro* differed from the axons of neurons which were developed *in vivo* (Banker and Cowan 1979).

The specific conditions, growth substrate, culture media and cell density, chosen for the culture will affect the survival (Okuda *et al.* 1994; Xie *et al.* 2000) as well as the growth and development of the cells in culture (Banker and Cowan 1979). The conditions may even alter the characteristics of the cells being cultured as has been demonstrated with bone marrow stromal cells in culture (Neuhuber et al. 2008) and with the SH-SY5Y cell line (Buttiglione *et al.* 2007).

These negative effects have been reduced in this study by quick, efficient culture preparation to minimise cell stress, using optimal media for these cells and a

short culture time to avoid the effect of changing cell characteristics over long periods in culture.

6.3.2. Other potential limitations

In the same way that a 2D culture cannot completely mimic a 3D environment it is also not possible to accurately reproduce a disease condition. For example, the problems associated with some models of oxidative stress are detailed in Chapter 4, Section 4.2.1. Moreover, while the treatment of cultures with $A\beta_{1-42}$ allows the investigation of effects on $A\beta_{1-42}$-induced toxicity and much information may be gained from these studies these cultures are missing the elements of the inflammation and NFT that occur in AD.

However, the very fact that these models only encompass one element of the disease at a time means that one can study individual factors without complications from interference and in this way these models do allow the further understanding of effects and interactions of compounds on cells or effects between compounds.

Another potential limitation of this study is that Western blot experiments are only semi-quantitative. Despite this limitation, this type of experiment appears in this study at least to be the simplest and clearest way to examine changes in protein levels.

6.4. Future work

There are some additional points that could not be included in this work but should be investigated to directly follow up the results of the experiments that were carried out in this study. These are detailed below.

1) CLN was seen to have a trophic effect at 100ng/ml in low density cultures and investigation into the mechanism of this trophic effect is necessary. Microarray analysis on TR146 cells showed that CLN can up-regulate the expression of PDGF (Szaniszlo *et al.* 2009) so it would be reasonable to hypothesise that this may translate into increased protein levels *in vitro* and CLN may increase the levels of PDGF or other growth factors in culture media. This may be studied by analysis of the release of soluble factors into the cell culture media in the presence of CLN.

2) Analyses of whether the changes seen in SOD1 protein levels correspond with changes in enzyme activity and also if these *in vitro* effects are also seen *in vivo*. I would hypothesise that there would not be changes in SOD1 activity upon treatment with Aβ as the activity levels of this enzyme have been shown not to increase in AD patients but there is some disagreement in the literature on this point as discussed in Chapter 1, Section 1.4.3. Moreover oestrogen has been found to directly cause an increase in the expression of SOD2 (Borras *et al.* 2005) and CLN was found to increase protein levels of SOD1 in B50 cells and in previous work was shown to increase the expression of antioxidant enzymes in TR146 cells (Szaniszlo *et al.* 2009). Therefore another suggestion for further work is an investigation into the effects of CLN on protein levels and activity of SOD2 in primary cells or *in vivo*.

3) Investigation of changes in Cdk5 activity in response to bovine CLN and $A\beta_{1-42}$. $A\beta_{1-42}$ has been demonstrated to cause an increase in the activity of Cdk5 that relates to toxicity (Orellana *et al.* 2006) and therefore CLN may be hypothesised to prevent this increase in activity despite having no effect on protein levels in primary hippocampal cells or B50 cells. Further to this, analysis should be done on the effect of CLN on cytokine release in primary hippocampal cells to establish whether the hypothesised mode of action of CLN via TNFα release to decrease Cdk5 protein levels or activity is correct.

4) CLN did not significantly affect the expression of activated caspase 3 in either the primary hippocampal cells or B50 cells but there are other points in apoptotic initiation and signalling that CLN may affect. Therefore studying the effects of CLN on other apoptotic markers such as caspase 8, annexin V or TUNEL labelling would be worthwhile follow up work. Moreover CLN may prevent the Aβ mediated affects on the levels of apoptotic control molecules, pro-apoptotic Bcl-w, or anti-apoptotic Bcl-2, in the same way that oestrogen has been found to (Yao *et al.* 2007).

CLN has been well studied and its effects are now becoming more fully understood. There is however much more that can be done in our detailed analysis of these effects which does not directly follow on from the experiments carried out in this study. There are many other potential mechanisms of action for the beneficial effects of CLN that should be investigated in future work *in vitro* or *in vivo*. Some of these are outlined below.

1) CLN has been shown using microarrays on TR146, buccal mucosal cells to decrease the expression of PKA which regulates Tau phosphorylation (Szaniszlo *et al.* 2009) and the levels of PKA protein and phosphorylated Tau should be analysed to assess whether the gene expression change observed translates to protein levels changes and ultimately a prevention of Tau phosphorylation. There has not however been any study to directly investigate the effects of CLN on Tau phosphorylation and aggregation. Western blotting may be used to examine changes in the levels of phosphorlyated Tau in cell lysate upon treatment with CLN and Aβ and one would hypothesise that there would be a reduction in Tau phosphorylation with CLN treatment.

2) As discussed in Chapter 1, Section 1.1.2 the role of GSK3β in mediating the toxicity of Aβ has been well documented (Hooper *et al.* 2008). Furthermore compounds that can prevent the activation of GSK3β have been found to prevent the toxicity of Aβ as detailed in Chapter 1, Section 1.1.3.2. From this work and the work discussed in Section 1, Section 1.6.4 showing that CLN can alter the expression of kinases (Szaniszlo *et al.* 2009) it could be hypothesised that CLN would also be able to prevent the Aβ induced increase in GSK3β activity and in this way dramatically decrease Aβ induced toxicity.

3) The immune system is affected in AD as discussed in Chapter 1, Section 1.1.2 and this may contribute to the initiation and progression of the disease. The effects of CLN on the status of the immune system and immune system cells have been very well documented as described in Chapter 1, Section 1.6.2. These two factors have not yet been tested together and no study has been done to investigate the effects of CLN on immune system activity in AD patients.

4) The long term, 16 month, clinical trials gave CLN orally to the patients (Leszek *et al.* 1999) yet there has not been a study to investigate the mechanism by which CLN exerts effects in the CNS. It appears from this study and previous work by others that the effects of CLN may be at least partially mediated by antioxidant effects and therefore is indirect and we now know many of the effects that CLN may have on cells *in vitro*. However, an important part of our understanding of CLN involves knowledge of what tissues it has it has access to and may therefore act upon *in vivo.* CLN as a whole is too large to cross the blood–brain barrier although there may be a small active component, such as the nonapeptide, which is able to cross in the brain. Moreover, analysis of how well CLN is absorbed in the gut and into adipose tissue would greatly add to the work that has already been carried out using CLN.

5) It has been shown that CLN is able to prevent and even reverse the aggregation of $A\beta_{1\text{-}40}$ *in vitro* (Schuster *et al.* 2005). However, the action of CLN on the production of Aβ has not been investigated. A transgenic APP cell line would allow this to be studied. Furthermore, the effect of CLN on the production and aggregation of Aβ *in vivo* has not yet been examined. Transgenic animals would allow the opportunity for these effects to be studied.

6) The ultrastructural changes in the brain upon treatment with Aβ are now well established (Watt *et al.* 1994; Richardson *et al.* 2003; Kelly and Ferreira 2007) and we have shown that CLN alone does not cause any significant ultrastructural changes in hippocampal cells in culture. Further studies should now be carried out using electron microscopy to investigate the ability of CLN to prevent the

Aβ-induced ultrastructural changes in synapses in the rat brain or on primary cells in culture.

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Appendix

8.1. Materials

Company or source	Reagent/Consumable
Gibco/Invitrogen	FCS, N2 supplement, EBSS, B27 supplement, HS and BME.
Sigma-Aldritch	DMEM, glutamine, Trypsin-EDTA, Penicillin-streptomycin, Papain, Cysteine, chicken egg white Trypsin inhibitor, Glucose, Sodium pyruvate and RIPA lysis buffer.
Corning	Culture flasks
Greiner	Culture plates as well as other plastics
VWR	Glass coverslips
Agar Scientific	Citifluor mountant
Regen Therapeutics	Bovine CLN, $A\beta_{1-42}$
Biosource	$A\beta_{1-42}$
Promega	The MTS cytotoxicity assay kit
Kindly donated by Kathleen Wright and James Phillips	DCFH-DA from Sigma-Aldritch
Biorad	DC protein assay
National diagnostics	Protogel and Tris/glycine, Tris/glycine/SDS buffers
Perkin-Elma	ECL developing reagents
GE healthcare	Full range rainbow protein marker

8.2. Antibodies

8.2.1. Primary antibodies

Cell type/epitope	Antibody	Company
Neurons	Mouse monoclonal anti-MAP-2 raised against rat brain MAPs	Abcam (ab11267)
Astrocytes	Rabbit polyclonal anti-GFAP antibody raised against GFAP from cow spinal cord	Dako-Cytomation (20334)
Oligodendrocytes	Mouse monoclonal antibodies against CNPase raised against the full length human protein	Abcam (ab6319)
Microglia	Mouse monoclonal antibody against CD11b raised against rat macrophages	Abcam (ab1211)
SOD1	Rabbit anti-SOD1 antibody raised against residues on synthetic human SOD1	Abcam (ab51254)
Cdk5	Rabbit polyclonal antibody against Cdk5 raised against the C-terminus of human Cdk5	Santa Cruz (SC-173)
Activated caspase 3	Rabbit polyclonal antibody against activated caspase 3 raised against a synthetic peptide conjugated to KLH derived from within residues 150 - 250 of human active caspase 3	Abcam (ab13847)
Actin	Rabbit monoclonal antibody against modified β-cytoplasmic actin N-terminal peptide, Ac-Asp-Asp-Asp-Ile-Ala-Ala-Leu-Val-Ile-Asp-Asn-Gly-Ser-Gly-Lys.	Sigma-aldritch (A1978)

8.2.2. Secondary antibodies

Antibody	Company
anti-rabbit biotinylated	GE Healthcare (RPN1004V1)
strepavidin fluorescein	GE Healthcare (RPN123V1)
Goat anti-mouse AMCA conjugated	Millipore (AP130M)
Goat anti-mouse Rhodamine conjugated	Millipore (AP124R)
Anti-rabbit Horseraddish peroxidise conjugated	Thermo Scientific -Pierce (31460)